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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 29 January 2001 (29.01.01)	
International application No. PCT/US99/07745	**Applicant's or agent's file reference** 00246/233WO1
International filing date (day/month/year) 08 April 1999 (08.04.99)	**Priority date** (day/month/year)
Applicant MCMAHON, Andrew, P. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

08 November 2000 (08.11.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

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RECD 30 MAR 2001
PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
21508-033		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/07745	08 APRIL 1999	NONE
International Patent Classification (IPC) or national classification and IPC IPC(7): C07K 14/475 and US Cl.: 530/350		
Applicant PRESIDENT AND FELLOWS OF HARVAD COLLEGE		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08 NOVEMBER 2000	07 MARCH 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Bao Qun Li Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/07745

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-44 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 45-47 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets~~/fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/07745

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-20	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-20	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-20	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-20 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the method of stimulating kidney tubule formation utilizing Wnt polypeptide.

---------- NEW CITATIONS ----------

NONE

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
00246/233W01		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 07745	08/04/1999	
Applicant		
INDUCTION OF KIDNEY TUBULE FORMATION.et.al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmittnd to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   INDUCTION OF KIDNEY TUBULE FORMATION

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIO SEARCH REPORT

International application No.
[redacted]T/US 99/07745

Box I Observations wh re certain claims were found unsearchabl (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
US 99/07745

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/475 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KISPERT A ET AL: "Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney." DEVELOPMENT, (1998 NOV) 125 (21) 4225-34. , XP002130717 page 4226, column 1, paragraph 4 -column 2, paragraph 1 page 4227, column 1, paragraph 4 -page 4228, column 2, paragraph 3; table 2 page 4230, column 2, paragraph 1 --- -/--	1,9-11, 13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2000	01/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Charles, D

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]US 99/07745

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STARK K ET AL: "Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4." NATURE, (1994 DEC 15) 372 (6507) 679-83. , XP002130718 page 679, column 2, paragraph 2 page 682, column 1, paragraph 1	1,10,11
A	WO 98 06747 A (GENENTECH INC) 19 February 1998 (1998-02-19) page 5, line 6 - line 15; claim 16 page 5, line 26 - line 37	1,2,7,8
A	US 5 686 289 A (HUMES H DAVID ET AL) 11 November 1997 (1997-11-11) column 1, line 13 - line 18; claims 1,5,7 column 5, line 16 - line 32 column 10, line 14 - line 16 column 10, line 53 - line 59	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/07745

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9806747 A	19-02-1998	US 5851984 A	22-12-1998
		AU 3911297 A	06-03-1998
		CA 2262469 A	19-02-1998
		EP 0918794 A	02-06-1999
US 5686289 A	11-11-1997	US 5549674 A	27-08-1996
		AU 692191 B	04-06-1998
		AU 7970194 A	08-05-1995
		CA 2173594 A	27-04-1995
		EP 0746343 A	11-12-1996
		JP 9503941 T	22-04-1997
		WO 9511048 A	27-04-1995

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/06, 5/08

A1

(11) International Publication Number: **WO 99/57248**

(43) International Publication Date: 11 November 1999 (11.11.99)

(21) International Application Number: PCT/US98/08716

(22) International Filing Date: 30 April 1998 (30.04.98)

(71) Applicant *(for all designated States except US)*: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: McMAHON, Andrew, P. [GB/US]; 128 Kendall Road, Lexington, MA 02173 (US). LEE, Scott, K. [US/US]; 54 Chestnut Street, Cambridge, MA 02139 (US). TAKADA, Shinji [-/-]; -

(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: INDUCTION OF NEURONAL REGENERATION

(57) Abstract

An enriched population of mammalian dorsal neural progenitor cells, e.g., dopaminergic neural precursor cells, are described that are useful to induce neuronal regeneration in mammals suffering from a neurodegenerative disease.

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INDUCTION OF NEURONAL REGENERATION

Background of the Invention

The invention relates to neuronal growth and differentiation.

Wnt polypeptides are secreted cysteine-rich glycosylated polypeptides that play a role in the development of a wide range of organisms. The Wnt family of polypeptides contains at least 16 mammalian members which bind to an extracellular domain of a family of cell surface proteins called Frizzled receptors. Wnt polypeptides may play a role in embryonic induction, generation of cell polarity, and specification of cell fate. Deregulation of Wnt signalling has been linked to tumor development.

Summary of the Invention

The invention is based on the discovery that Wnt polypeptides regulate neuronal precursor cell fate, i.e., the type of neuron into which a precursor cell differentiates depends qualitatively on the Wnt signal it receives. For example, Wnt-1 specifies midbrain cell fate. In addition to regulation of cell type, Wnt polypeptides regulate neural precursor state, i.e., proliferation versus differentiation. A stem cell phenotype is characterized by mitotic activity and a lack of characteristics associated with a mature terminally-differentiated neuron, whereas a differentiated phenotype is characterized by a lack of proliferation and acquisition of properties, e.g., morphology or cell surface proteins, associated with a particular terminally-differentiated neural cell type.

The invention features an enriched population of mammalian dorsal neural precursor cells that maintain a stem cell phenotype in the presence of a Wnt polypeptide. By an "enriched population" is meant a population of

cells that has been treated with a Wnt polypeptide to selectively expand a desired neural precursor cell type. Thus, an enriched population of neural precursor cells is not naturally-occurring, but contains a higher concentration of neural precursor cells having a particular cell fate compared to the concentration in a naturally-occurring population of stem cells.

The Wnt polypeptide is preferably a Wnt-1 class polypeptide such as Wnt-1, Wnt-2, Wnt-3a, Wnt-7a, and Wnt-7b. A Wnt-1 class polypeptide is a Wnt polyeptide that transforms C57MG cells in culture. Other Wnt polypeptides, e.g., Wnt-5a, that play a role in midbrain development may also be used to culture stem cells.

A drawback of conventional stem cell preparations is that they heterogenous, i.e., a precursor cell with a particular cell fate may constitute only a small fraction of the population. The invention solves this problem by providing a method of selecting for a desired precursor cell type, i.e., by contacting the cell with a Wnt polypeptide. For example, the invention provides a method of treating a heterogeneous population of neural cell precursor cells to enrich for neural precursor cells committed to a desired cell fate by culturing the population with a Wnt polypeptide, e.g., a Wnt-1 class polypeptide. Neural precursor cells selectively proliferate in the presence of the Wnt polypeptide, whereas other precursor cells do not proliferate (or proliferate at a rate lower than that of the dorsal neural precursor cells). Thus, repeated culturing of the population in this manner yields a population of neural precursor cells that is progressively more enriched for dorsal neural precursor cells. The enriched population of dorsal neural precursor cells is at least 60%, preferably at least 75%, more preferably at least 80%, more preferably at least 90%, more preferably at least

95%, more preferably at least 98%, and most preferably 100% dorsal neural precursor cells.

For example, the invention encompasses an enriched population of mammalian dopaminergic neuron precursor cells. Selection of such cells is accomplished by contacting a heterogenous population of precursor cells with a Wnt-1 class polypeptide. The cells may be human or porcine cells and may be derived from fetal tissue. The cells are mitotically-active and maintaining a stem cell phenotype in the presence of a Wnt polypeptide. In the absence of a Wnt polypeptide, the cells cease proliferating and differentiate into dopaminergic neurons. A dopaminergic neuron is one that produces dopamine. Preferably, the Wnt polypeptide is human Wnt-1 or a fragment of Wnt-1 that is capable of stimulating proliferation of such cells and arresting differentiation. Since Wnt polypeptides have mitogenic activity for neural precursor cells, a method of stimulating cell proliferation of a dorsal neural precursor cell is carried out by contacting the cell in culture or *in vivo* with a Wnt-1 polypeptide and/or a Wnt-3a polypeptide. To promote proliferation of mammalian dopaminergic neuron precursor cells, the polypeptide preferably has a sequence that is at least 80% identical to amino acid sequence of naturally-occurring human Wnt-1 (SEQ ID NO:1) and has a biological property of naturally-occurring Wnt-1, e.g., the ability to maintain the neural stem cell phenotype of a neural precursor cell in culture.

Table 1: Human Wnt-1 amino acid sequence

1 MGLWALLPGW VSATLLLALA ALPAALAANS SGRWWGIVNV ASSTNLLTDS
KSLQLVLEPS
61 LQLLSRKQRR LIRQNPGILH SVSGGLQSAV RECKWQFRNR RWNCPTAPGP
HLFGKIVNRG
121 CRETAFIFAI TSAGVTHSVA RSCSEGSIES CTCDYRRRGP GGPDWHWGGC
SDNIDFGRLF
181 GREFVDSGEK GRDLRFLMNL HNNEAGRTTV FSEMRQECKC HGMSGSCTVR
TCWMRLPTLR
241 AVGDVLRDRF DGASRVLYGN RGSNRASRAE LLRLEPEDPA HKPPSPHDLV
YFEKSPNFCT
301 YSGRLGTAGT AGRACNSSSP ALDGCELLCC GRGHRTRTQR VTERCNCTFH
WCCHVSCRNC
361 THTRVLHECL (SEQ ID NO:1)

Table 2: Human Wnt-2 amino acid sequence

MNAPLGGIWLWLPLLLTWLTPEVNSSWWYMRATGGSSRVMCDNV
PGLVSSQRQLCHRHPDVMRAISQGVAEWTAECQHQFRQHRWNCNTLDRDHSLFGRVLL
RSSRESAFVYAISSAGVVFAITRACSQGEVKSCSCDPKKMGSAKDSKGIFDWGGCSDN
IDYGIKFARAFVDAKERKGKDARALMNLHNNRAGRKAVKRFLKQECKCHGVSGSCTLR
TCWLAMADFRKTGDYLWRKYNGAIQVVMNQDGTGFTVANERFKKPTKNDLVYFENSPD
YCIRDREAGSLGTAGRVCNLTSRGMDSCEVMCCGRGYDTSHVTRMTKCGCKFHWCCAV
RCQDCLEALDVHTCKAPKNADWTTAT (SEQ ID NO:2)

Where a particular polypeptide or nucleic acid molecule is said to have a specific percent identity to a reference polypeptide or nucleic acid molecule of a defined length, the percent identity is relative to the reference polypeptide or nucleic acid molecule. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Sequence identity can be measured using sequence analysis software (for example, the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705), with the default parameters as specified therein.

An enriched population of mammalian dorsal hindbrain precursor cells is also within the invention. Such cells are selected by contacting a heterogenous population of cells with a mixture of a Wnt-1 polypeptide and a Wnt-3a polypeptide. An enriched population of mitotically-active mammalian hippocampal neuron precursor cells are selected by culturing the cells in the presence of a Wnt-1 class polypeptide such as Wnt-3a; the cells maintain a stem cell phenotype in culture in the presence of a Wnt-3a polypeptide. Such precursor cells cease proliferating and differentiate into hippocampal neurons in the absence of the Wnt-3a polypeptide. Preferably, the Wnt-3a polypeptide has a sequence that is at least 80% identical to SEQ ID NO:2 and has a biological property of naturally-occurring Wnt-3a, e.g., the ability to maintain a neural stem cell phenotype in culture.

Table 3: Murine Wnt-3a amino acid sequence

MAPLGYLLVLCSLKQALGSYPIWWSLAVGPQYSSLSTQPILCAS
IPGLVPKQLRFCRNYVEIMPSVAEGVKAGIQECQHQFRGRRWNCTTVSNSLAIFGPVL
DKATRESAFVHAIASAGVAFAVTRSCAEGSAAICGCSSRLQGSPGEGWKWGGCSEDIE
FGGMVSREFADARENRPDARSAMNRHNNEAGRQAIASHMHLKCKCHGLSGSCEVKTCW
WSQPDFRTIGDFLKDKYDSASEMVVEKHRESRGWVETLRPRYTYFKVPTERDLVYYEA
SPNFCEPNPETGSFGTRDRTCNVSSHGIDGCDLLCCGRGHNARTERRREKCHCVFHWC
CYVSCQECTRVYDVHTCK (SEQ ID NO:3)

Table 10: Human Wnt-3a amino acid sequence

CKCHGLSGSC EVKTCWWSQP DFRAIGDFLK DKYDSASEMV VEKHRESRGW
VETLRPRYTY FKVPTERDLV YYEASPNFCE PNPETGSFGT RDRTCNVSSH
GIDGCDLLCC GRGHNARAER RREKCRCVFH WCC (SEQ ID NO:10)

Table 4: Human Wnt-7a amino acid sequence

1 MNRKALRCLG HLFLSLGMVC LRIGGFSSVV ALGATIICNK IPGLAPRQRA ICQSRPDAII
61 VIGEGSQMGL DECQFQFRNG RWNCSALGER TVFGKELKVG SRDGAFTYAI IAAGVAHAIT
121 AACTHGNLSD CGCDKEKQGQ YHRDEGWKWG GCSADIRYGI GFAKVFVDAR EIKQNARTLM
181 NLHNNEAGRK ILEENMKLEC KCHGVSGSCT TKTCWTTLPQ FRELGYVLKD KYNEAVHVEP
241 VRASRNKRPT FLKIKKPLSY RKPMDTDLVY IEKSPNYCEE DPVTGSVGTQ GRACNKTAPQ

301 ASGCDLMCCG RGYNTHQYAR VWQCNCKFHW CCYVKCNTCS ERTEMYTCK

Table 5: Human Wnt-7b partial amino acid sequence

1 GVSGSCTTKT CWTTLPKFRE VGHLLKEKYN AAVQVEVVRA SRLRQPTFLR IKQLRSYQKP
61 METDLVYIEK SPNYCEEDAA TGSVGTQGRI CNRTSPGADG CDTMCCGRGY NTHQYTKVWQ
121 CNCK (SEQ ID NO:5)

Table 6: Human Wnt-5a amino acid sequence

1 MAGSAMSSKF FLVALAIFFS FAQVVIEANS WWSLGMNNPV QMSEVYIIGA QPLCSQLAGL
61 SQGQKKLCHL YQDHMQYIGE GAKTGIKECQ YQFRHRRWNC STVDNTSVFG RVMQIGSRET
121 AFTYAVSAAG VVNAMSRACR EGELSTCGCS RAARPKDLPR DWLWGGCGDN IDYGYRFAKE
181 FVDARERERI HAKGSYESAR ILMNLHNNEA GRRTVYNLAD VACKCHGVSG SCSLKTCWLQ
241 LADFRKVGDA LKEKYDSAAA MRLNSRGKLV QVNSRFNSPT TQDLVYIDPS PDYCVRNEST
301 GSLGTQGRLC NKTSEGMDGC ELMCCGRGYD QFKTVQTERC HCKFHWCCYV KCKKCTEIVD
361 QFVCK (SEQ ID NO:6)

Other patterning signals, e.g., Bmp polypeptides or Hedgehog polypeptides, are also used to induce differentiation of an enriched population of neural precursor cells into a differentiated neural cell type.

An population of neural precursor cells that is enriched for a particular type of precursor cell is useful clinically, e.g., to repopulate a depleted population of a particular type of neuron. Depletion of subpopulations of neurons may be the result of the progression of a neurodegenerative disease such as Parkinson's Disease, Amyotrophic Lateral Sclerosis, Diffuse Lewy Body Disease, Cortical-basal Ganglionic Degeneration, Hallervorden-Spatz Disease, or Myoclonic Epilepsy. A method of inducing neuronal regeneration in an adult mammal suffering from a neurodegenerative disorder is carried out by transplanting into the affected mammal an enriched population of dorsal neural precursor cells such as that cultured in the presence of one or more Wnt polypeptides. To promote proliferation of the transplanted stem cells *in vivo*, the method may also include a step of administering to the mammal a Wnt polypeptide or Wnt agonist systemically or locally at the site of transplantation. For example, a patient suffering from Parkinson's disease is treated by transplanting into the brain of the patient an enriched

population of dopaminergic neuron precursor cells. A Wnt-1 polypeptide may be administered concurrently or subsequent to transplantation.

The invention also includes a transgenic non-human mammal, e.g., a rodent such as a mouse, the germ cells and somatic cells of which contain a null mutation, e.g., a deletion, in DNA encoding a Wnt polypeptide. These animals can serve as useful models of neural development. By "null mutation" is meant an alteration in the nucleotide sequence that renders the gene incapable of expressing a functional protein product. The mutation could be in a Wnt gene regulatory region or in the coding sequence. It can, e.g., introduce a stop codon that results in production of a truncated, inactive gene product or it can be a deletion of all or a substantial portion of the coding sequence.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The invention provides methods of selecting for neural precursor cells that will differentiate into a particular type of neuron upon exposure to a differentiation-inducing condition or composition and methods for growing such precursor cells in culture. The methods permit expansion of precursor cells of a desired cell fate to achieve large number of cells suitable for clinical transplantation.

Neural Stem Cells

Primary neural progenitor cells are obtained from a mammalian source, e.g., fetal CNS precursor tissue such as developing neural crest tissue, using known methods. Such primary cells are cultured in the presence of a Wnt polypeptide such as Wnt-1 class polypeptide (purified from a natural source or produced recombinantly) in

conventional tissue culture medium such as Dulbecco's Modified Eagles Medium (DMEM) containing fetal calf serum or in serum-free tissue culture medium.

Wnt polypeptides regulate neuronal precursor cell fate as well as neural precursor state. Wnt polypeptides that belong to the Wnt-1 class of Wnt polypeptides are preferably used to culture neural precursor cells for the purpose of maintaining a stem cell phenotype and promoting proliferation. A Wnt-1 class polypeptide is a Wnt polyeptide and that transforms C57MG cells in culture. To determine whether a Wnt polypeptide is a Wnt-1 class polypeptide, C57MG cells (an epithelial cell line derived from normal mouse mammary tissue) are cultured in the presence and absence of the Wnt polypeptide using known methods, e.g., that described by Wong et al., 1994, Mol. Cell. Biol. 14:6278-6286, and their morphology assessed for a transformed phenotype. Normal C57MG cells grow in a monolayer with a regular, cuboidal appearance at confluence, whereas culturing C57MG cells in the presence of a Wnt-1 class polypeptide causes the cells to become transformed, i.e., refractile and elongated, growing over other cells in a disorganized manner. Wnt polypeptides of the Wnt-1 class cause C57MG cells to assume a transformed phenotype. Human Wnt polypeptides which belong to the Wnt-1 class include Wnt-1 (GENBANK Accession #139743, Wnt-2 (GENBANK Accession #139750), Wnt-3a, Wnt-7a (GENBANK Accession #2501663), and Wnt-7b (GENBANK Accession #546573). A Wnt polypeptide, e.g., human Wnt-5a (GENBANK Accession #731157), that is not a member of the Wnt-1 class may also be used (with or without a Wnt-1 class polypeptide) to culture neural precursor cells.

The cells are cultured in the presence or absence of feeder cells. Feeder cells may be engineered to produce a recombinant Wnt-1 class polypeptide such as

Wnt-1 and/or Wnt-3a, e.g., by introducing DNA encoding a Wnt polypeptide, e.g., DNA encoding Wnt-1, Wnt-2, Wnt-3a, Wnt-7a or Wnt-7b, into the cell and culturing the cell under conditions that permit expression of the recombinant polypeptide and secretion of the polypeptide into the extracellular environment. For example, feeder cells can be transfected with an expression vector containing DNA having the sequence of naturally-occuring Wnt-1, Wnt-2, or Wnt-3a.

Table 7: Human Wnt-1 Nucleotide Sequence

```
        1 atgtatgtat gtatgtatgt atgtatgtat acgtgcgtgc acctgtgtgt
gcttggtgtc
       61 agtggggctc agacatcacc tgattccctg gaactggagt tacaggtggc
tataagccac
      121 cacttgggtg ctgagaacag agtccgggcc tctggcagag cagtcagtgc
ttttagccac
      181 tgagccactc tcatcccccc aattatgttc atcttgagtt gggcaggtac
ggtggcggaa
      241 taggcctgta atcccagcag tcactggacc atcatgggtt ctacatatta
aacctttatg
      301 ttaggtaggg tcacacagca agatccggtc acaaaaccag caacaacaaa
aaccaaaagg
      361 agccagcttc ttcccacaag cattctttcc ctcaggtctt cagctccatc
tgacagctac
      421 tcggctggtg gtcctatcct ttctgagcct agttgccaga gaaacaagcc
cggttcatct
      481 tcatgactag cacatctaat gataagcaca ggttgactca aggtgccata
gagtgacact
      541 aggtacccag agcgacagaa tgacacctat gagtgcacgt cgttaatcac
aaacacacac
      601 acacacacac acacacacac acacacacac tcatgcaccc acctgcaaac
acaattgcag
      661 ccttctggac gtctcctgtc acagccccac ctccttcctg atacactgcg
ttaagtggtg
      721 actgtaacaa aatgacttca tgctctccct gtcctgagcc aaattacaca
attatttgga
      781 aagggctcaa aatgttcttc gttagaagtt tctggataca ccaatacaca
ggagcgtgca
      841 ccctcagaac acatgtacac tttgacttaa tctcacgggt gacacaccga
cgcttacact
      901 ccccctagcc cacagaggca aactgctggg cgcttctgag tttctcactg
ccaccagctc
      961 ggtttgctca gcctaccccc gcaccccgcg cccgggaatc cctgaccaca
gctccaccca
     1021 tgctctgtct ccttcttttc cttctctgtc cagccgtcgg ggttcctggg
tgaggaagtg
     1081 tctccacgga gtcgctggct agaaccacaa ctttcatcct gccattcaga
atagggaaga
     1141 gaagagacca cagcgtaggg gggacagagg agacggactt cgagaggaca
gccccaccgg
     1201 cgcgtgtggg ggaggcaatc caggctgcaa acaggttgtc cccagcgcat
tgtccccgcg
     1261 ccccctggcg gatgctggtc cccgacgggc tccggacgcg cagaagagtg
aggccggcgc
     1321 gcgtgggagg ccatcccaag gggaggggtc ggcggccagt gcagacctgg
aggcggggcc
```

1381 accaggcagg gggcgggggt gagccccgac ggttagcctg tcagctcttt gctcagaccg
1441 gcaagagcca cagcttcgct cgccactcat tgtctgtggc cctgaccagt gcgccctggt
1501 gcttttagtg ccgcccgggc ccggaggggc agcctcttct cactgcagtc agcgccgcaa
1561 ctataagagg cctataagag gcggtgcctc ccgcagtggc tgcttcagcc cagcagccag
1621 gacagcgaac catgctgcct gcggcccgcc tccagactta ttagagccag cctgggaact
1681 cgcatcactg ccctcaccgc tgtgtccagt cccaccgtcg cggacagcaa ccacagtcgt
1741 cagaaccgca gcacagaacc agcaaggcca ggcaggccat ggggctctgg gcgctgctgc
1801 ccagctgggt ttctactacg ttgctactgg cactgaccgc tctgcccgca gccctggctg
1861 ccaacagtag tggccgatgg tggtaagtga gctagtacgg ggtccgccac ttgtcctggg
1921 gcaaagagcc aggcacgggc cttacccagc tcccacgctg tggggatcac caacctacag
1981 acccccctcg tgcattgtga cttcacatcc agggtgctca cacctagaac tagctctgct
2041 gaagtggggc acatcattgg catgcagaag cccagataca ccaggctcag agaccattcc
2101 catttaatac gaccccgttt ctgctgagca acaggtccca acctcgctgt ggtgggtgct
2161 caggtgtccc ttaggtcttg aaccaaaaaa aaaaaaaaaa aaaaaaaaaa accagatatt
2221 agctttgagg tgagggagtg gaattcctaa gtttttcaag gtgggcaagg ctgcaggtgg
2281 ggtttctcct cgggggctga cttgaagaaa ggaagagcta aggtagccat gccttttctg
2341 tccactcact agactctgga gctcagggcc aggcaaggat agggtggtac agcctgtatg
2401 gttaggatgc aggtcccctc ccctggactg aacccttatg catcccgcca ggggcatcgt
2461 gaacatagcc tcctccacga acctgttgac ggattccaag agtctgcagc tggtgctcga
2521 gcccagtctg cagctgctga gccgcaagca gcggcgactg atccgacaga acccggggat
2581 cctgcacagc gtgagtggag ggctccagag cgctgtgcga gagtgcaaat ggcaattccg
2641 aaaccgccgc tggaactgcc ccactgctcc ggggccccac ctcttcggca agatcgtcaa
2701 ccgaggtggg tgcccaggaa agcgacgctt ccgggattaa gggaaaagca gggtcatctc
2761 cagggcatag gcgggcgaag gcagggaaga catcccaggg ttatatgtga tcaaactgag
2821 aatcgcctgg tgccggcagt taccgtaggt cagcaccaga ttctttctag ccttgcgttg
2881 tgagcatgat ctttaacgtt gctggccact ggcccacaga aagggaattc cggatcgtgg
2941 gcgctgggcg acagctgttt ttccctagcc ttcctcaaag gtacctggga agctgatctc
3001 tgagggctag ctagggttgt gcttcgcacc cagcaaagtt tgcactgcca atactagtag
3061 cgatcttggc tatgcagatt tgttctactt gggaatctcc ccttggagct gctctgctag
3121 ggctctggag tctcagtaaa gcttagagag gagggcattc catgcttcgc acacatgact
3181 ccaaggatgt tggactgtag ggtaccaagt cttccaaaca gggtgctgag ttggccccac
3241 gccttctctc aactgatgcg gggtcgcttc acccacaggc tgccgagaaa cagcgttcat
3301 cttcgcaatc acctccgccg gggtcacaca ttccgtggcg cgctcctgct ccgaaggctc

3361 catcgagtcc tgcacctgcg actaccggcg gcgcggccct gggggccccg actggcactg
3421 ggggggctgc agtgacaaca tcgattttgg tcgcctcttt ggccgagagt tcgtggactc
3481 cggggagaag gggcgggacc tacgcttcct catgaacctt cacaacaacg aggcagggcg
3541 aacggtacgt cggtgtgtcc ggaaccaatg gcaggggaga tgtaagacag gtgcacgggg
3601 acagaggcac agggaggggc ttcccgagag agtgggactc taggagggaa gacagagaag
3661 aggtggtggt tgagggcaaa gaggttcctg agctgatgac agaacagaag agattagcag
3721 gctatcaaca cgtgggatgt attgagatgg ctccatggca cacttttgaa agataaaagt
3781 gacttgctgg cgtggagcag agtctggccg aatgtcccta tctcagcggg ccattttgca
3841 cttcctctct cccgagctta gtcacacctg gaccttggct gaagtttcca cagcatcgac
3901 gtgacccggg tggggtgggg gtggggaagt atgggtggtg gttcgtggga tgttggcttt
3961 gacctttttct tccctcctcc cctcgtcccc tcctcccccа gaccgtgttc tctgagatgc
4021 gccaagagtg caaatgccac gggatgtccg gctcctgcac ggtgcgcacg tgttggatgc
4081 ggctgcccac gctgcgcgct gtgggcgacg tgctgcgcga ccgcttcgac ggcgcctccc
4141 gcgtccttta cggcaaccga ggcagcaacc gcgcctcgcg ggcggagctg ctgcgcctgg
4201 agcccgaaga ccccgcgcac aagcctccct cccctcacga cctcgtctac ttcgagaaat
4261 cgcccaactt ctgcacgtac agtggccgcc tgggcacagc tggcacagct ggacgagctt
4321 gcaacagctc gtctcccgcg ctggacggct gtgagctgct gtgctgtggc cgaggccacc
4381 gcacgcgcac gcagcgcgtc acggagcgct gcaactgcac cttccactgg tgctgccacg
4441 tcagctgccg caactgcacg cacacgcgcg ttctgcacga gtgtctatga ggtgccgcgc
4501 ctccgggaac gggaacgctc tcttccagtt ctcagacaca ctcgctggtc ctgatgtttg
4561 cccaccctac cgcgtccagc cacagtccca gggttcatag cgatccatct ctcccacctc
4621 ctacctgggg actcctgaaa ccacttgcct gagtcggctc gaacccttt gccatcctga
4681 gggccctgac ccagcctacc tccctccctc tttgagggag actccttttg cactgccccc
4741 caatttggcc agagggtgag agaaagattc ttcttctggg gtgggggtgg ggaggtcaac
4801 tcttgaaggt gttgcggttc ctgatgtatt ttgcgctgtg acctctttgg gtattatcac
4861 ctttccttgt ctctcgggtc cctataggtc ccttgagttc tctaaccagc acctctgggc
4921 ttcaaggcct ttcccctccc acctgtagct gaagagtttc cgagttgaaa gggcacggaa
4981 agctaagtgg gaaaggaggt tgctggaccc agcagcaaaa ccctacattc tccttgtctc
5041 tgcctcggag ccattgaaca gctgtgaacc atgcctccct cagcctcctc ccacccctc
5101 ctgtcctgcc tcctcatcac tgtgtaaata atttgcaccg aaatgtggcc gcagagccac
5161 gcgttcggtt atgtaaataa aactatttat tgtgctgggt tccagcctgg gttgcagaga
5221 ccaccctcac cccacctcac tgctcctctg ttctgctcgc cagtcctttt gttatccgac
5281 ctttttttctc ttttacccag cttctcatag gcgcccttgc ccaccggatc agtatttcct

5341 tccactgtag ctattagtgg ctcctcgccc ccaccaatgt agtatcttcc tctgaggaat
5401 aaaatatcta tttttatcaa cgactctggt ccttgaatcc agaacacagc atggcttcca
5461 acgtcctctt cccttccaat ggacttgctt ctcttctcat agccaaacaa aagagataga
5521 gttgttgaag atctcttttc cagggcctga gcaaggaccc tgagatcctg acccttggat
5581 gaccctaaat gagaccaact agggatc (SEQ ID NO:7)

Table 8: Human Wnt-2 Nucleotide Sequence

1 agcagagcgg acgggcgcgc gggaggcgcg cagagctttc gggctgcagg cgctcgctgc
61 cgctggggaa ttgggctgtg ggcgaggcgg tccgggctgg cctttatcgc tcgctgggcc
121 catcgtttga aactttatca gcgagtcgcc actcgtcgca ggaccgagcg gggggcgggg
181 gcgcggcgag gcggcggccg tgacgaggcg ctcccggagc tgagcgcttc tgctctgggc
241 acgcatggcg cccgcacacg gagtctgacc tgatgcagac gcaagggggt taatatgaac
301 gcccctctcg gtggaatctg gctctggctc cctctgctct tgacctggct caccccgag
361 gtcaactctt catggtggta catgagagct acaggtggct cctccagggt gatgtgcgat
421 aatgtgccag gcctggtgag cagccagcgg cagctgtgtc accgacatcc agatgtgatg
481 cgtgccatta gccagggcgt ggccgagtgg acagcagaat gccagcacca gttccgccag
541 caccgctgga attgcaacac cctggacagg gatcacagcc tttttggcag ggtcctactc
601 cgaagtagtc gggaatctgc ctttgtttat gccatctcct cagctggagt tgtatttgcc
661 atcaccaggg cctgtagcca aggagaagta aaatcctgtt cctgtgatcc aaagaagatg
721 ggaagcgcca aggacagcaa aggcattttt gattggggtg gctgcagtga taacattgac
781 tatgggatca aatttgcccg cgcatttgtg gatgcaaagg aaaggaaagg aaaggatgcc
841 agagccctga tgaatcttca caacaacaga gctggcagga aggctgtaaa gcggttcttg
901 aaacaagagt gcaagtgcca cggggtgagc ggctcatgta ctctcaggac atgctggctg
961 gccatggccg acttcaggaa aacgggcgat tatctctgga ggaagtacaa tggggccatc
1021 caggtggtca tgaaccagga tggcacaggt ttcactgtgg ctaacgagag gtttaagaag
1081 ccaacgaaaa atgacctcgt gtattttgag aattctccag actactgtat cagggaccga
1141 gaggcaggct ccctgggtac agcaggccgt gtgtgcaacc tgacttcccg gggcatggac
1201 agctgtgaag tcatgtgctg tgggagaggc tacgacacct cccatgtcac ccggatgacc
1261 aagtgtgggt gtaagttcca ctggtgctgc gccgtgcgct gtcaggactg cctggaagct
1321 ctggatgtgc acacatgcaa ggcccccaag aacgctgact ggacaaccgc tacatgaccc
1381 cagcaggcgt caccatccac cttcccttct acaaggactc cattggatct gcaagaacac
1441 tggacctttg ggttctttct gggggatat ttcctaaggc atgtggcctt tatctcaacg
1501 gaagcccccc cttcctcccc gggggcccca ggatgggggg ccacacgctg cacctaaagc
1561 ctaccctatt ctatccatct cctggtgttc tgcagtcatc tccccctcctg gcgagttctc
1621 tttggaaata gcatgacagg ctgttcagcc gggagggtgg tgggcccaga ccactgtctc
1681 cacccacctt gacgtttctt ctttctagag cagttggcca agcagaaaaa aaagtgtctc
1741 aaaggagctt tctcaatgtc ttcccacaaa tggtcccaat taagaaattc catacttctc
1801 tcagatggaa cagtaaagaa agcagaatca actgcccctg acttaacttt aacttttgaa
1861 aagaccaaga cttttgtctg tacaagtggt tttacagcta ccacccttag ggtaattggt
1921 aattacctgg agaagaatgg ctttcaatac ccttttaagt ttaaaatgtg tatttttcaa
1981 ggcatttatt gccatattaa aatctgatgt aacaaggtgg ggacgtgtgt cctttggtac
2041 tatggtgtgt tgtatctttg taagagcaaa agcctcagaa agggattgct ttgcattact
2101 gtccccttga tataaaaaat ctttagggaa tgagagttcc ttctcactta gaatctgaag
2161 ggaattaaaa agaagatgaa tggtctggca atattctgta actattgggt gaatatggtg
2221 gaaaataatt tagtggatgg aatatcagaa gtatatctgt acagatcaag aaaaaaagga
2281 agaataaaat tcctatatca t (SEQ ID NO:8)

Table 9: Murine Wnt-3A Nucleotide Sequence

1 gaattcatgt cttacggtca aggcagaggg cccagcgcca ctgcagccgc gccacctccc
61 agggccgggc cagcccaggc gtccgcgctc tcggggtgga ctccccccgc tgcgcgctca
121 agccggcgat ggctcctctc ggatacctct tagtgctctg cagcctgaag caggctctgg
181 gcagctaccc gatctggtgg tccttggctg tgggacccca gtactcctct ctgagcactc
241 agcccattct ctgtgccagc atcccaggcc tggtaccgaa gcagctgcgc ttctgcagga

301 actacgtgga gatcatgccc agcgtggctg agggtgtcaa agcgggcatc caggagtgcc
361 agcaccagtt ccgaggccgg cgttggaact gcaccaccgt cagcaacagc ctggccatct
421 ttggccctgt tctggacaaa gccacccggg agtcagcctt tgtccatgcc atcgcctccg
481 ctggagtagc tttcgcagtg acacgctcct gtgcagaggg atcagctgct atctgtgggt
541 gcagcagccg cctccagggc tccccaggcg agggctggaa gtggggcggc tgtagtgagg
601 acattgaatt tggaggaatg gtctctcggg agtttgccga tgccagggag aaccggccgg
661 atgcccgctc tgccatgaac cgtcacaaca atgaggctgg gcgccaggcc atcgccagtc
721 acatgcacct caagtgcaaa tgccacgggc tatctggcag ctgtgaagtg aagacctgct
781 ggtggtcgca gccggacttc cgcaccatcg gggatttcct caaggacaag tatgacagtg
841 cctcggagat ggtggtagag aaacaccgag agtctcgtgg ctgggtggag accctgaggc
901 cacgttacac gtacttcaag gtgccgacag aacgcgacct ggtctactac gaggcctcac
961 ccaacttctg cgaacctaac cccgaaaccg gctccttcgg gacgcgtgac cgcacctgca
1021 atgtgagctc gcatggcata gatgggtgcg acctgttgtg ctgcgggcgc gggcataacg
1081 cgcgcactga gcgacggagg gagaaatgcc actgtgtttt ccattggtgc tgctacgtca
1141 gctgccagga gtgcacacgt gtctatgacg tgcacacctg caagtaggag agctcctaac
1201 acgggagcag ggttcattcc gaggggcaag gttcctacct gggggcgggg ttcctacttg
1261 gaggggtctc ttacttgggg actcggttct tacttgaggg cggagatcct acctgtgagg
1321 gtctcatacc taaggacccg gtttctgcct tcagcctggg ctcctatttg ggatctgggt
1381 tccttttag gggagaagct cctgtctggg atacgggttt ctgcccgagg gtggggctcc
1441 acttggggat ggaattccaa tttgggccgg aagtcctacc tcaatggctt ggactcctct
1501 cttgacccga cagggctcaa atggagacag gtaagctact ccctcaacta ggtggggttc
1561 gtgcggatgg gtgggagggg agagattagg gtccctcctc ccagaggcac tgctctatct
1621 agatacatga gagggtgctt cagggtgggc cctatttggg cttgaggatc ccgtgggggc
1681 ggggcttcac cccgactggg tggaactttt ggagaccccc ttccactggg gcaaggcttc
1741 actgaagact catgggatgg agctccacgg aaggaggagt tcctgagcga gcctgggctc
1801 tgagcaggcc atccagctcc catctggccc ctttccagtc ctggtgtaag gttcaacctg
1861 caagcctcat ctgcgcagag caggatctcc tggcagaatg aggcatggag aagaactcag
1921 gggtgatacc aagacctaac aaaccccgtg cctgggtacc tcttttaaag ctctgcaccc
1981 cttcttcaag ggctttccta gtctccttgg cagagctttc ctgaggaaga tttgcagtcc
2041 cccagagttc aagtgaacac ccatagaaca gaacagactc tatcctgagt agagagggtt
2101 ctctaggaat ctctatgggg actgctagga aggatcctgg gcatgacagc ctcgtatgat
2161 agcctgcatc cgctctgaca cttaatactc agatctcccg ggaaacccag ctcatccggt
2221 ccgtgatgtc catgccccaa atgcctcaga gatgttgcct cactttgagt tgtatgaact

2281 tcggagacat ggggacacag tcaagccgca gagccagggt tgtttcagga cccatctgat
2341 tccccagagc ctgctgttga ggcaatggtc accagatccg ttggccacca ccctgtcccg
2401 agcttctcta gtgtctgtct ggcctggaag tgaggtgcta catacagccc atctgccaca
2461 agagcttcct gattggtacc actgtgaacc gtccctcccc ctccagacag gggaggggat
2521 gtggccatac aggagtgtgc ccggagagcg cggaaagagg aagagaggct gcacacgcgt
2581 ggtgactgac tgtcttctgc ctggaacttt gcgttcgcgc ttgtaacttt attttcaatg
2641 ctgctatatc cacccaccac tggatttaga caaaagtgat tttctttttt ttttttttctt
2701 ttctttctat gaaagaaatt attttagttt atagtatgtt tgtttcaaat aatggggaaa
2761 gtaaaaagag agaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaa
(SEQ ID NO:9)

Table 11: Human Wnt-3a nucleotide sequence

tgtaagtgcc acgggctgtc gggcagctgc gaggtgaaga catgctggtg
gtcgcaaccc gacttccgcg ccatcggtga cttcctcaag gacaagtacg
acagcgcctc ggagatggtg gtggagaagc accgggagtc ccgcggctgg
gtggagaccc tgcggccgcg ctacacctac ttcaaggtgc ccacggagcg
cgacctggtc tactacgagg cctcgcccaa cttctgcgag cccaaccctg
agacgggctc cttcggcacg cgcgaccgca cctgcaacgt cagctcgcac
ggcatcgacg gctgcgacct gctgtgctgc ggccgcggcc acaacgcgcg
agcggagcgg cgccgggaga agtgccgctg cgtgtttcac tggtgctgt
(SEQ ID NO:11)

Stem cells may be obtained from a a heterologous donor animal such as a pig. The animal is euthanized and tissue removed using a sterile procedure. Brain areas of particular interest include any area from which progenitor cells can be obtained which will serve to restore function to a degenerated area of the host's brain. These regions include areas of the CNS including the cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the peripheral nervous system (PNS) including the carotid body and the adrenal medulla. For example, cells may be obtained from the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell groups such as the globus pallidus, the subthalamic nucleus, or the substantia nigra pars compacta (which is found to be degenerated in Parkinson's Disease patients).

Human heterologous neural progenitor cells may be derived from fetal tissue obtained from elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, in particular during epilepsy surgery, and more particularly during temporal lobectomies and hippocampalectomies.

Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Dissociation can be obtained using any known procedure, including treatment with enzymes, e.g., trypsin or collagenase, or by using physical methods of dissociation such as with a blunt instrument. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose. Low Ca^{2+} aCSF contains the same ingredients except for $MgCl_2$ at a concentration of 3.2 mM and $CaCl_2$ at a concentration of 0.1 mM.

Dissociated cells can be placed into any culture medium capable of supporting cell growth, including MEM, DMEM, RPMI, F-12. The medium may containin supplements which support cellular metabolism such as glutamine and other amino acids, vitamins, minerals and proteins such as transferrin. In some cases, the medium may contain bovine, equine, chicken or human serum. A preferable medium for neural precursor cells is a mixture of DMEM and F-12. Conditions for culturing mimic physiological conditions, e.g., physiological pH, preferably between pH 6-8, more preferably close to pH 7, even more particularly about pH 7.4 at a temperature that is at or close to physiological temperature.

Cells can be grown in suspension or on a fixed substrate, but proliferation of the precursor cells is preferably done in suspension to generate large numbers of cells by formation of "neurospheres" (see, for example, Reynolds et al., 1992, Science 255:1070-1709; and PCT Publications WO93/01275, WO94/09119, WO94/10292, and WO94/16718). Cell suspensions in culture medium are supplemented with any growth factor which allows for the proliferation of precursor cells and seeded in any receptacle capable of sustaining cells, preferably in culture flasks or roller bottles. Cells typically proliferate within 3-4 days in a 37°C incubator, and proliferation can be reinitiated at any time after that by dissociation of the cells and resuspension in fresh medium containing growth factors.

In the absence of substrate, cells lift off the floor of the flask and continue to proliferate in suspension forming a hollow sphere of undifferentiated cells. After approximately 3-10 days *in vitro*, the proliferating clusters (neurospheres) are fed every 2-7 days, and more particularly every 2-4 days by gentle centrifugation and resuspension in medium containing a Wnt polypeptide or a growth factor.

After 6-7 days *in vitro*, individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, more particularly by titrating the neurospheres with a pipette. Single cells from the dissociated neurospheres are suspended in culture medium containing growth factors, and differentiation of the cells can be induced by plating (or resuspending) the cells in the presence of a Wnt agonist, and (optionally) any other factor capable of inducing and/or sustaining differentiation.

The tissue culture media is supplemented with a Wnt polypeptide (either by adding a Wnt polypeptide to

the culture media or by adding feeder cells producing a Wnt polypeptide) to maintain a stem cell phenotype of the precursor cells and to promote proliferation of the cells. Other commercially available growth factors such as Fibroblast Growth Factor (FGF) or Epidermal Growth Factor (EGF) are added to the culture as mitogens.

Cells cultured in the presence of a Wnt polypeptide, e.g., a member of the Wnt-1 class of polypeptides, proliferate and maintain a stem cell phenotype. Differentiation of the cells can proceed upon the removal of the Wnt polypeptide and/or addition of a composition that promotes differentiation.

A naturally-occurring population of neural crest cells contains multipotent (i.e., uncommitted) neural crest cells and committed precursor cells. The role of Wnt proteins employed in the present method is to culture a population of neural precursor cells, e.g., a naturally-occurring population of neural crest cells, (1) to induce cell fate of an uncommitted precursor and thereby give rise to a committed precursor cell and (2) to maintain such cells in a stem cell state (e.g., to arrest the development of a committed precursor cell towards becoming a terminally-differentiated neuronal cell). For example, the present method can be used *in vitro* to induce and/or maintain the differentiation of neural crest cells into glial cells, schwann cells, chromaffin cells, cholinergic sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The Wnt protein can be used alone, or can be used in combination with other neurotrophic factors which act to more particularly enhance a particular differentiation fate of the neuronal precursor cell. In the later instance, an Wnt polypeptide might be viewed as ensuring that the treated cell has achieved a particular phenotypic state such that the cell is poised

along a certain developmental pathway so as to be properly induced upon contact with a secondary neurotrophic factor. Even relatively undifferentiated stem cells or primitive neuroblasts can be maintained in culture and caused to differentiate by treatment with Wnt agonists. Exemplary primitive cell cultures comprise cells harvested from the neural plate or neural tube of an embryo.

A population of neural precursor cells is characterized as having a stem cell phenotype when the level of proliferation of the cells in standard tissue culture media (e.g., MEM, DMEM, RPMI, F-12) in the presence of a Wnt polypeptide is at least 20% greater than the level of proliferation in the same tissue culture media without the Wnt polypeptide. Preferably, the level of cell proliferation is at least 50% greater in the presence of a Wnt polypeptide compared to the level of proliferation in the absence of a Wnt polypeptide. Proliferation is measured using known methods, e.g, incorporation of tritiated thymidine. Neural cells with a differentiated phenotype are characterized as non-proliferating and having the physical characteristics and cell markers of a mature terminally-differentiated neuron.

Primary stem cells may be immortalized by a variety of known techniques such as retrovirus-mediated transduction of an immortalizing gene, e.g., avian *myc* (*v-myc*).

Graft preparation

The therapeutic methods of the invention which utilize enriched populations of neural precursor cells may be used to treat neurodegenerative diseases and other types of diseases that result in depletion of neural cells. In addition to chronic depletion associated with progressive neurodegenerative diseases, neurons may be

killed as a consequence of infectious diseases, autoimmune diseases, and immunodeficiency diseases. Clinical outcome of treatment can be assessed by measuring as motor and cognitive capabilities of the patient, length of patient survival, quality of life.

Precursor cells cultured in the presence of a Wnt polypeptide as described above are washed and resusupended in a pharmaceutically acceptable excipient, e.g., a solution of 0.6% glucose-saline, are transplanted into brain tissue of a recipient mammal using known methods, e.g., those described by Gage et al., 1987, Ciba Found. Symp. 126:143-159. A small volume of a cell suspension is steriotaxically injected into a desired region, e.g., the hippocampus, of a mammal. For example, approximately 10^5 cells are infused into a desired location of the brain of the patient over 30 min.

Subsequent to transplantation, a Wnt polypeptide may be administered to the patient to induce further proliferation of stem cell *in vivo*. Wnt polypeptides can be administered in the form of a nerve prostheses for the repair of central and peripheral nerve damage. In particular, where a crushed or severed axon is intubulated by use of a prosthetic device, Wnt polypeptides can be added to the prosthetic device to increase the rate of growth and regeneration of the dendritic processes.

Alternatively, prior to transplantation, the cells may be exposed to a composition that induces differentiation Treatment of neurodegenerative disease

Neurodegenerative diseases include familial and sporadic amyotrophic lateral sclerosis (FALS and ALS, respectively), familial and sporadic Parkinson's disease, Huntington's disease, familial and sporadic Alzheimer's disease, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse lewy

body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, gilles de la tourette syndrome, and Hallervorden-Spatz disease. Most of the diseases are typified by onset during the middle adult years and lead to rapid degeneration of specific subsets of neurons within the neural system, ultimately resulting in premature death. There is no known cure nor is there an effective therapy to slow the progression for any of the listed diseases.

Parkinson's disease (paralysis agitans) is a common neurodegenerative disorder which appears in mid to late life. Familial and sporadic cases occur, although familial cases account for only 1-2 percent of the observed cases. The neurological changes which cause this disease are somewhat variable and not fully understood. Patients frequently have nerve cell loss with reactive gliosis and Lewy bodies in the substantia nigra and locus coeruleus of the brain stem. Similar changes are observed in the nucleus basalis of Meynert. Nigrostriatal dopaminergic neurons are most affected.

The disorder generally develops asymmetrically with tremors in one hand or leg and progresses into symmetrical loss of voluntary movement. Eventually, the patient becomes incapacitated by rigidity and tremors. In the advanced stages the disease is frequently accompanied by dementia.

Diagnosis of both familial and sporadic cases of Parkinson's disease can only be made after the onset of the disease. Anticholinergic compounds, propranolol, primidone and levodopa are frequently administered to modify neural transmissions and thereby suppress the symptoms of the disease, though there is no known therapy which halts or slows the underlying progression. The therapeutic methods described herein may be administered

in conjunction with existing therapeutic approaches to neurodegenerative diseases.

The death of the dopaminergic neurons in the basal ganglia is an underlying defect of this progressive chronic disease as the basal ganglia are involved in the control of voluntary movements. Wnt-polypeptides and neural precursor cells cultured in the presence of Wnt polypeptides, e.g., Wnt-1, are useful in the treatment of Parkinson's disease and other disorders of midbrain dopamine circuitry. Transplantation of dopaminergic neural precursor cells is used to repopulate a patient's depleted population of dopaminergic neurons to treat or ameliorate the symptoms of Parkinson's disease.

Another neurodegenerative disease, Alzheimer's disease, can take two forms: disease exist: presenile dementia, in which the symptoms emerge during middle age, and senile dementia which occurs in the elderly. Both forms of the disease appear to have the same pathology. Diseases which affect learning and memory may be characterized by a depletion of hippocampal cells. Transplantation of hippocampal neural precursor cell is used to repopulate a patient's depleted population of hippocampal neurons to treat neurodegenerative diseases that affect learning and memory such as Alzheimer's disease.

Example 1: Wnt Signaling and Proliferation

Wnt signalling was found to regulate the expansion of dorsal neural precursors. Wnt-1 and Wnt-3a are coexpressed at the dorsal midline of the developing neural tube. Wnt-1 is involved in midbrain patterning, and Wnt-3a is involved in the formation of the paraxial mesoderm. The absence of a dorsal neural tube phenotype in animals with a mutation in either gene suggested that Wnt signalling is redundant. The data described below indicate that in the absence of both Wnt-1 and Wnt-3a,

there is a marked deficiency in neural crest derivatives, which originate from the dorsal neural tube, and a pronounced reduction in dorsolateral precursors within the neural tube itself.

Mice lacking both Wnt-1 and Wnt-3a signaling were generated. Mice which are heterozygous for null alleles of Wnt-1 and Wnt-3a were made using known methods (e.g., McMahon et al., 1990, Cell 62:1073-1085 and Takada et al., 1994, Genes Dev. 8:174-189). Compound heterozygotes (on a predominantly 129/Sv background) were intercrossed to recover compound mutants. Genotypes were confirmed by genomic Southern hybridization and polymerase chain reaction (PCR). Whole mount immunostaining was carried out using antibodies specific for neurofilaments, CRABP-1, and Lmx-1b. Skeletons from 18.5 d.p.c embryos were prepared and stained with alcian blue and alizarin red using known methods.

To evaluate cell proliferation and death, embryos were collected at 9.5 d.p.c (20-25 somite stage development) after intraperitoneal injection of pregnant females with 50 μg per body weight of 5-bromo-2'-deoxyuridine (BrdU). Mice were killed one hour later. Embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. After dehydration, wax embedding and sectioning at a thickness of 6 μm, serial sections were dewaxed and either stained with haematoxylin and eosin, or assayed for BrdU incorporation for apoptotic death using a standard TUNEL procedure.

Compound homozygotes were recovered at the expected Mendelian frequency (51 compound homozygotes in 673 embryos. The frequency was close to the expected frequency of 1/16) between 9.0 and 10.5 days post coitum (d.p.c.). Due to the termination of caudal axial development accompanying the loss of Wnt-3a activity,

relatively few of these embryos survived to 18.5 d.p.c. (3 compound homozygotes in 151 embryos).

To assess the development of the dorsal neural tube in compound mutants, neural crest derived structures were examined. Neural crest cells are among the first differentiated cell types to be formed by dorsal neural precursors. Neurofilament staining indicated that both neural crest derived cranial and spinal ganglia formation were unaltered in single mutants (either Wnt-1 or Wnt-3a mutants) which were either wild type or heterozygous for mutations in the other Wnt member. However, in double mutants, neurons derived from the proximal ganglion of cranial nerve IX (glossopharyngeal), which is formed by crest cells originating from rhombomere 6 within the hindbrain (r6), were absent. In contrast, the distal ganglion which is placodal in origin was present. In addition, there was a marked reduction in the proximal axons of cranial nerves V (trigeminal, r2 derived) and X (vagus, r7 derived). Similarly, in the trunk, there was a reduction in neurofilament staining in the cervical dorsal root ganglia. Further, cell counts indicated a 60% decrease in the cellular content of the dorsal root ganglia. Whole-mount *in situ* hybridization with probes specific for *Islet-1* and cadherin-6, markers of neuronal and glial neural crest derivatives, respectively, confirmed the reduction or absence of crest cells within the cranial ganglia and dorsal root ganglia. In contrast sympathetic ganglia, which express *c-ret*, were unaffected.

The reduction of neurogenic and gliogenic crest derivatives in the caudal head and rostral trunk regions indicates that fewer neural crest cells emerge in embryos lacking both Wnt-1 and Wnt-3a signaling. The issue of neural crest formation was evaluated by examining CRABP-1 immunoreactivtity and AP-2 transcription. CRABP-1 is

normally present in the dorsal CNS at 9.0 d.p.c. as well as in migrating neural crest cells arising from r2, 4 and 6. AP-2 is first expressed at 8.5 d.p.c. in the dorsal neural plate, coincident with neural crest formation. By 9.5 d.p.c. cranial expression is absent in the neural tube but persists in migrating and maturing neural crest derivatives at cranial and spinal cord levels. Loss of function studies have demonstrated that AP-2 is essential for development of neural crest derived structures. A clear decrease was observed in migrating CRABP-1 positive cells within the hindbrain, although CRABP-1 staining within the CNS appeared to be relatively normal. Similarly, examination of AP-2 expression revealed a reduction in both cranial and trunk neural crest. In contrast to their wild type litter mates, double mutants also retained AP-2 expression within the dorsal CNS at 9.5 d.p.c. Thus, in the absence of Wnt-1 and Wnt-3a, there is both a reduction in neural crest cell formation and persistent expression of AP-2 at the dorsal midline.

To determine whether Wnt-signaling was required throughout the period of cranial crest formation, expression of TRP-2 was evaluated. TRP-2 is a marker of presumptive melanocytes which are dominant in late formed cranial crest derivatives. At 11.5 d.p.c., TRP-2 expression was virtually absent within presumptive melanocytes migrating within the hindbrain region of double mutants though a few TRP-2 cells remained at the dorsal midline. In view of the prolonged expression of AP-2 within the dorsal CNS, TRP-2 expressing cells may be differentiating at a later stage, or they may be retained at the midline because Wnt-signaling promotes neural crest migration. Neither CRABP-1, TRP-2 or AP-2 expression was altered in the forebrain indicating that there is regional specificity in the requirement for these Wnt-signals.

Much of the head skeleton is generated by cranial neural crest. Distinct skeletal elements are derived from neural crest cells which emerge from different regions of the brain. To determine whether the reduction in neural crest formation in double mutants leads to alterations in the skeleton, 18.5 d.p.c. embryos were stained with alcian blue and alizarin red to examine cartilage and bone development. The stapes and the main body of the hyoid bone including the greater horn which originate from crest cells derived from r3-5 and r6-7, respectively, were absent. Thyroid cartilage showed a consistent dysmorphology. The reduction in hindbrain crest formation was also reflected in the absence of specific skeletal derivatives. In contrast, despite the early loss of forebrain, midbrain and rostral hindbrain in double mutants, the development of skeletal crest derivatives from these regions, as well as non-crest derived bones, was largely normal though there was some reduction in development of the squamosal, alisphenoid, basisphenoid, presphenoid and otic capsule. These data indicate that, in the absence of Wnt-1/3a signaling, several neural crest cell fates form, but there is a dramatic reduction in neural crest derivatives in the hindbrain region and in the spinal cord.

Neural crest cell development, and other aspects of dorsal polarity within the developing CNS, are thought to be regulated by BMP signals produced initially by the dorsal ectoderm and subsequently by the dorsal CNS. BMP-7 expression was induced, as expected, in the roof plate of double mutants. The data indicate that it was unlikely that defective neural crest development resulted from a secondary loss of BMP-signaling within the dorsal neural tube.

To determine whether Wnt-signaling directly regulates dorso-ventral polarity within the CNS, the

distribution of a number of regionally expressed markers was examined. Whereas spinal cord levels appeared normal, the hindbrain displayed a striking phenotype. Expression of Wnt-3a, Wnt-1 and Lmx-1b was normal in the roof plate. Thus, unlike other aspects of Wnt-signaling in the mammalian embryo, these Wnt-expressing cells did appear to require the Wnt-signals they produce. In contrast, expression of Math1 (which is activated at 9.5 d.p.c. in cells immediately adjacent to the roof plate) and Pax-3 (which occupies most of the dorsal half of the CNS) were dramatically reduced in the double mutant hindbrain. Dbx expression at the dorsal-ventral interface and Pax-6 expression in the ventro-lateral CNS were normal.

The data indicate that in the hindbrain, Wnt-signaling does not appear to play a role directly in the primary patterning processes which lead to the establishment of distinct cell fates in appropriate positions along the dorsoventral axis. Rather, it appears to play an essential role in the subsequent expansion of dorso-lateral neural progenitors. In support of a potential role in neural proliferation, transgenic analysis demonstrated that Wnt-1 can act as a potent mitogen when ectopically expressed within the dorsal CNS.

In normal development there is a ventral to dorsal progression in the formation of different neural crest derivatives. In the double mutants, the most severely affected crest derivatives were more proximal (dorsally located) structures. The stapes was absent from the second branchial arch while the lesser horn of the hyoid was unaltered, and in the trunk, dorsal root ganglia were markedly reduced while the sympathetic ganglia appeared normal. If the signals governing commitment to neural crest cell fates were unperturbed in the double mutant,

but renewal of a multipotential dorsal neural progenitor pool required Wnt-signals, the expected result would be a loss of later forming neural crest derivatives in Wnt-1/-3a mutants, as precursors within the neural tube became limiting.

Cell proliferation and cell death in hindbrain tissue sections (9.5 d.p.c; 20-25 somites) were analyzed using BrdU incorporation and TUNEL staining, respectively. Dorsal neural precursors were reduced, but no discernible change was detected in either proliferation or cell death within remaining dorsal regions of Wnt-1 and Wnt-3a mutants. As these two Wnts are first coexpressed at the otic level when the first neural crest cells appear (at about 8.5 d.p.c; 8-10 somites), it is likely that the main reduction in dorsolateral neural precursors occurs between 8.5 and 9.5 d.p.c.

These data indicate that Wnt signalling regulates dorsoventral patterning in the mammalian CNS through the control of cell proliferation.

Example 2: Wnt-3A Signaling in Neuronal Differentiation

Wnt-3a expression in the mouse begins in the primitive streak region of the late egg cylinder at 7.5 d.p.c. and is maintained in the tail bud until tail formation is complete. To determine which cell types in the primitive streak region express Wnt-3a, the expression of Wnt-3a transcripts was examined in wild type embryos at the 7 somite stage. Expression was detected in the ectoderm layer in the primitive streak region but was absent from the node. Expression was further restricted for ventrally located cells in the anterior streak region. In contrast, in the posterior streak, most cells in the ectoderm layer expressed Wnt-3a. Wnt-3a expression was not observed in migrating mesodermal cells at either anterior or posterior

positions. These data indicate that Wnt-3a expression is localized to the primitive ectoderm prior to the physical segregation of the paraxial mesoderm and is downregulated as cells ingress through the primitive streak.

The phenotype of Wnt-3a homozygous mutant embryos was analyzed at early somite stages. At the 5 somite stage, no obvious differences in morphology between wild type and Wnt-3a mutant embryos were detected. However, by the 7 somite stage, differences in the shape of the primitive streak region were apparent. In Wnt-3a mutants, the width of the primitive streak region is narrower than in the wild type embryos and this phenotype becomes more pronounced by the 10 somite stage.

To further investigate the abnormal morphology of mutant embryo, histological analysis of the sections was carried out. In wild type embryos at the 7 somite stage, migrating presomitic mesodermal cells were observed under the primitive ectoderm layer in the primitive streak region. However, in Wnt-3a mutant embryos at the same stage, no migrating presomitic mesoderm cells were observed; in contrast, the shape and movement of cells ingressed through the primitive streak are quite different from those in normal embryos. In the anterior streak region of the mutant embryos, the ingressing cells were round in appearance, quite distinct from the usual stellate mesenchymal morphology of the ingressing mesoderm. Furthermore, these cells contacted each other and formed an ectopic tubular structure under the primitive streak at more posterior level. This tubular structure was not observed anterior to the streak where somites are present. Thus, in Wnt-3a mutant embryos, the absence of somite precursors appears to be correlated with the appearance of an ectopic tubular structure arising in the primitive streak region.

To identify the molecular characteristics of the ectopic tubular structure in Wnt-3a mutant embryos, *in situ* hybridization and whole mount immunostaining and the expression of a variety of molecular markers detected.

MF-1, encodes a forkhead domain containing protein, which is normally expressed in somites, presomitic mesoderm, and lateral mesoderm at 9.5 d.p.c. In Wnt-3a mutant embryos at this stage, no obvious MF-I expression was observed in the position where the ectopic tube was formed posterior to the forelimb level. A transverse section of the stained embryo at this axial level clearly indicated that no MF-1 transcripts were localized in the ectopic tube. Similarly another paraxial mesoderm marker, Mox-1, was not expressed in the ectopic tube in Wnt-3a mutants at either 8.5 or 9.5 d.p.c. The data indicate that the ectopic tube does not have the molecular and morphological characteristics of paraxial mesoderm.

Mash-I is normally expressed in central nervous system and peripheral nervous system precursors at 9.5 d.p.c. but not in the mesoderm. In Wnt-3a mutant embryos at the same stage, *Mash-1* expression was detected not only in these region but also in the region ventral to the original neural tube posterior to the forelimb level. A transverse section of Wnt-3a mutants at the axial level, where abnormal Mash-7 expression was observed, indicated that the ventral expression of Mash-I was localized in the ectopic tube. A second neural marker, HES-5, which is normally expressed in CNS, was also expressed in the ectopic tube in Wnt-3a mutants at 9.5 d.p.c. To explore further whether neurons differentiate in the ectopic tube, Wnt-3a mutant embryos at 10.5 d.p.c. were immunostained with antineurofilament antibody, 2H3. Neurofilament expressing cells were present in both the dorsal neural tube and the ectopic ventral tube.

The ectopic tube also exhibited polarity typical of CNS tissue. For example, Sonic hedgehog (Shh) is normally expressed in the floor plate of the neural tube. In 9.5 d.p.c. Wnt-3a mutant embryos, the notochord was present under the ventral ectopic tubular structure but not under the original neural tube at the axial level just posterior to the forelimbs while no notochord was absorbed at more posterior levels. Shh was expressed in ventrally in the ectopic tube where it contacts the notochord, suggesting, that the ectopic tube forms a floor plate in response to a Shh signaling by the notochord. The ectopic neural tube also exhibits dorsal polarity typical of the CNS such as the expression of the dorsal midline marker, Wnt-1 and increased levels of Pax-3 expression, where the tube contacts the surface ectoderm. In addition, expression of a ventral CNS marker, Pax-6, was suppressed where the ectopic tube contacts the surface ectoderm. Taken together, the data indicate that the ectopic tubular structure in the mutants has the molecular and cellular characteristics of an ectopic neural tube and consequently the loss of Wnt-3a signaling results in the formation of CNS precursors at the expense of paraxial mesoderm.

The phenotype of Wnt-3a knock out mutant embryos at 9.5 d.p.c. indicated that Wnt-3a is essential for formation of somitic mesoderm caudal to first 7-9 somites. In the absence of somite formation, an ectopic tubular structure which displays both cellular and molecular characteristics of presumptive CNS tissue is formed. Several lines of evidences suggest that the neural tube was formed ectopically. First, transverse sections of Wnt-3a mutant embryos at an early somite stage indicated that cells delaminating from and ingressing through the primitive streak form an epithelial cell layer that contribute to an ectopic tube

under the primitive ectoderm in the primitive streak region. Second, the notochord contacts the ventral but not the dorsal neural tube, suggesting that the ventral (ectopic) neural tube had already formed at the time when the notochord was laid down. Third, by the analysis of serial transverse sections of several 8.5 and 9.5 d.p.c. Wnt-3a mutant embryos, it is apparent that the ectopic neural tube is not continuous with the original dorsal neural tube suggesting an independent origin.

The appearance of the ectopic neural tube correlates with the disappearance of the paraxial mesoderm precursors in Wnt-3a mutant embryos. This correlation suggests that the absence of Wnt-3a signaling in the primitive ectoderm of the primitive streak may lead to presumptive somitic mesoderm cells to adopting, neural cell fate. That is, a neural fate may be a "default" state for cells which normally give rise to both mesodermal and neural derivatives.

The results described herein indicate that in the normal primitive ectoderm, where Wnt-3a is expressed, undifferentiated cells can differentiate into both neural and somitic mesoderm cell lineages. At early somite stages, cells in the anterior primitive streak generate mostly somitic mesoderm, while cells in the posterior streak gives rise to mostly lateral mesoderm. In contrast, primitive ectoderm adjacent to the anterior primitive streak contributes mainly to somitic mesoderm and neuroectoderm, suggesting that these two cell types might arise from the same cell population. The data indicate that Wnt-3a signaling regulates cell fate specification between somitic mesoderm and neural lineages in the normal mouse embryo.

Although Wnt-3a is expressed in the anterior streak in regions which gives rise to somitic mesoderm, it is also expressed in more posterior regions which

generate lateral and ventral mesoderm. Thus, expression is not restricted to paraxial mesoderm precursors. Wnt-3a may establish a competence to respond to a paraxial mesoderm inducing signal, rather than itself directly inducing paraxial mesodermal cell fates. This competence may be broadly distributed within the streak.

Example 3: Wnt-1 signaling and mid-brain development

Expression of En-1 in the developing midbrain of Wnt-1 null embryos is sufficient to rescue midbrain and interior hindbrain development. In the mouse, Wnt-1 and Engrailed-1 (En-1) are first expressed in the presumptive midbrain, from 8.0 days post coitum (d.p.c.) and continue to be expressed, together with En-2, in overlapping patterns during midbrain development. In Wnt-$1^{-/-}$ (Wnt-1-null) embryos, En-1 and En-2 expression is initiated normally, but subsequently both domains of En expression are lost, which is concomitant with a failure of midbrain and anterior hindbrain development.

En-1 was expressed from the transgene WEXPZ-En-1 in a pattern similar to that of endogenous Wnt-1 gene. To assess whether En-1 was able to rescue the Wnt-1-null phenotype, embryos from matings of Wnt-$1^{+/-}$, WEXPZ-En-1^{+} males with Wnt-$1^{+/-}$ females were collected at 14.5 d.p.c., when the Wnt-$1^{-/-}$ phenotype can easily be scored morphologically. The genotype was subsequently determined by southern blotting. Wnt-$1^{+/+}$ and Wnt-$1^{+/-}$ embryos with or without WEXPZ-En-1 appeared to be wild-type (n = 112) whereas all Wnt-$1^{-/-}$ embryos (n = 12) displayed the Wnt-$1^{-/-}$ phenotype.

In Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos, 7 out of 17 appeared superficially wildtype, 8 out of 17 were partially rescued and only 2 out of 17 were similar to Wnt-$1^{-/-}$ embryos.

To characterize brain development in greater detail, a minimum of four embryos from each category were

sectioned for histological analysis. All Wnt-1$^{-/-}$ embryos lacked the midbrain and cerebellum. In contrast, in Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos that were scored as wild-type, the midbrain and cerebellum appeared similar to those of wild-type embryos. In partially rescued embryos, only the posterior midbrain and a slightly reduced cerebellum were apparent. The absence of rescue in some cases, and partial rescue in others, may reflect influences of the genetic background or variations in the levels of En-1 expressed from the transgene.

To characterize the development of the midbrain in Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos further, the expression of several genes normally transcribed in this region was examined at 10.5 d.p.c. Pax-5 is expressed in a broad domain at the midbrain-hindbrain junction, but this domain is missing in Wnt-1$^{-/-}$ embryos. In Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos, Pax-5 expression was detected in a pattern similar to that of the wild-type embryos. Wnt-1 and Fgf-8 are normally expressed in adjacent rings of cells just anterior and posterior to the midbrain-hindbrain junction, respectively. Fgf8 signalling is involved in midbrain development. In Wnt-1$^{-/-}$ embryos, both rings of expressing cells are absent. In contrast, both Wnt-1 and Fgf-8 were expressed in sharp rings of cells in Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos despite the fact that no morphologically obvious midbrain-hindbrain junction was apparent. These results indicate that Wnt-1 signaling at this later stage may not play a direct role in regulating Fgf-8 expression in adjacent cells. En gene expression was also restored in the mid-hindbrain region of Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos outside the area where the transgene is expressed.

In all the rescued embryos, the expression domains of Pax-5, Fgf-8, En, and, in a few cases, Wnt-1 were

slightly reduced relative to wild-type littermates (18 out
41 Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos expressed one of the markers examined, of these at least half were substantially rescued). One likely explanation is that rescued embryos have a smaller population of midbrain cells than wild-type siblings because when Wnt-1 and En-1 expression is initiated, Wnt-1 mRNA transcription is patchy, whereas En genes are expressed more uniformly in presumptive midbrain cells. Thus, in Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos, where En-1 is not uniformly expressed in all presumptive midbrain cells, only those cells that express En-1 at this early stage may contribute to midbrain development. As En-1 expression in the midbrain restores Fgf-8, Pax-5 and En expression in the anterior hindbrain, and subsequently cerebellum development in Wnt-1$^{-/-}$ embryos, the data suggest that a midbrain-derived signal other than Wnt-1 is necessary for anterior hindbrain development.

To assess whether expression of En-1 was sufficient to rescue the viability of Wnt-1$^{-/-}$ mice (pups are born but die within 24 h) pups were genotyped at 10 days post partum (n = 68). No live Wnt-1$^{-/-}$, WEXPZ-En-1^{+} mice were obtained indicating that En-1 was insufficient to rescue the Wnt-1-null phenotype completely. Further analysis indicated that between 14.5 and 18.5 d.p.c., brains of Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos deteriorate, indicating that there may be additional functions of Wnt-1 signaling that cannot be replaced by En-1. This conclusion is supported by analysis of two cranial motor nerves, III (oculomotor) and IV (trochlear), which normally develop adjacent to Wnt-1-expressing cells in the ventral midbrain. Each of these fail to develop in Wnt-1$^{-/-}$ embryos. Similarly, neither nerve forms in Wnt-1$^{-/-}$, WEXPZ-En-1^{+} embryos which have

global restoration of midbrain development. In contrast, a second ventral population, tyrosine-hydroxylase-expressing neurons (catecholaminergic neurons) of the substantia nigra, are rescued in Wnt-$1^{-/-}$, WEXPZ-En-1^{+} embryos.

These data demonstrate that, in the absence of a Wnt-1 signal, expression of En-1 from the Wnt-1 enhancer is sufficient to substantially rescue early midbrain and anterior hindbrain development, and suggest that a major role of Wnt-1 signalling in the mammalian brain is to maintain En expression.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: President and Fellows of Harvard College

(ii) TITLE OF INVENTION: INDUCTION OF NEURONAL REGENERATION

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: FastSEQ for Windows Version 2.0b

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US98/-----
(B) FILING DATE: 30-APR-1998

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Freeman, John W.
(B) REGISTRATION NUMBER: 29,066
(C) REFERENCE/DOCKET NUMBER: 00246/222WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/542-5070
(B) TELEFAX: 617/542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 370 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Gly Leu Trp Ala Leu Leu Pro Gly Trp Val Ser Ala Thr Leu Leu
 1               5                  10                  15
Leu Ala Leu Ala Ala Leu Pro Ala Ala Leu Ala Ala Asn Ser Ser Gly
            20                  25                  30
Arg Trp Trp Gly Ile Val Asn Val Ala Ser Ser Thr Asn Leu Leu Thr
        35                  40                  45
Asp Ser Lys Ser Leu Gln Leu Val Leu Glu Pro Ser Leu Gln Leu Leu
    50                  55                  60
Ser Arg Lys Gln Arg Arg Leu Ile Arg Gln Asn Pro Gly Ile Leu His
```

```
 65                  70                  75                  80
Ser Val Ser Gly Gly Leu Gln Ser Ala Val Arg Glu Cys Lys Trp Gln
                85                  90                  95
Phe Arg Asn Arg Arg Trp Asn Cys Pro Thr Ala Pro Gly Pro His Leu
            100                 105                 110
Phe Gly Lys Ile Val Asn Arg Gly Cys Arg Glu Thr Ala Phe Ile Phe
        115                 120                 125
Ala Ile Thr Ser Ala Gly Val Thr His Ser Val Ala Arg Ser Cys Ser
    130                 135                 140
Glu Gly Ser Ile Glu Ser Cys Thr Cys Asp Tyr Arg Arg Arg Gly Pro
145                 150                 155                 160
Gly Gly Pro Asp Trp His Trp Gly Gly Cys Ser Asp Asn Ile Asp Phe
                165                 170                 175
Gly Arg Leu Phe Gly Arg Glu Phe Val Asp Ser Gly Glu Lys Gly Arg
            180                 185                 190
Asp Leu Arg Phe Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Thr
        195                 200                 205
Thr Val Phe Ser Glu Met Arg Gln Glu Cys Lys Cys His Gly Met Ser
    210                 215                 220
Gly Ser Cys Thr Val Arg Thr Cys Trp Met Arg Leu Pro Thr Leu Arg
225                 230                 235                 240
Ala Val Gly Asp Val Leu Arg Asp Arg Phe Asp Gly Ala Ser Arg Val
                245                 250                 255
Leu Tyr Gly Asn Arg Gly Ser Asn Arg Ala Ser Arg Ala Glu Leu Leu
            260                 265                 270
Arg Leu Glu Pro Glu Asp Pro Ala His Lys Pro Pro Ser Pro His Asp
        275                 280                 285
Leu Val Tyr Phe Glu Lys Ser Pro Asn Phe Cys Thr Tyr Ser Gly Arg
    290                 295                 300
Leu Gly Thr Ala Gly Thr Ala Gly Arg Ala Cys Asn Ser Ser Ser Pro
305                 310                 315                 320
Ala Leu Asp Gly Cys Glu Leu Leu Cys Cys Gly Arg Gly His Arg Thr
                325                 330                 335
Arg Thr Gln Arg Val Thr Glu Arg Cys Asn Cys Thr Phe His Trp Cys
            340                 345                 350
Cys His Val Ser Cys Arg Asn Cys Thr His Thr Arg Val Leu His Glu
        355                 360                 365
Cys Leu
    370
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asn Ala Pro Leu Gly Gly Ile Trp Leu Trp Leu Pro Leu Leu Leu
  1                 5                  10                  15
Thr Trp Leu Thr Pro Glu Val Asn Ser Ser Trp Trp Tyr Met Arg Ala
            20                  25                  30
Thr Gly Gly Ser Ser Arg Val Met Cys Asp Asn Val Pro Gly Leu Val
        35                  40                  45
Ser Ser Gln Arg Gln Leu Cys His Arg His Pro Asp Val Met Arg Ala
    50                  55                  60
Ile Ser Gln Gly Val Ala Glu Trp Thr Ala Glu Cys Gln His Gln Phe
65                  70                  75                  80
Arg Gln His Arg Trp Asn Cys Asn Thr Leu Asp Arg Asp His Ser Leu
```

```
                85                  90                  95
Phe Gly Arg Val Leu Leu Arg Ser Ser Arg Glu Ser Ala Phe Val Tyr
            100                 105                 110
Ala Ile Ser Ser Ala Gly Val Val Phe Ala Ile Thr Arg Ala Cys Ser
        115                 120                 125
Gln Gly Glu Val Lys Ser Cys Ser Cys Asp Pro Lys Lys Met Gly Ser
    130                 135                 140
Ala Lys Asp Ser Lys Gly Ile Phe Asp Trp Gly Gly Cys Ser Asp Asn
145                 150                 155                 160
Ile Asp Tyr Gly Ile Lys Phe Ala Arg Ala Phe Val Asp Ala Lys Glu
                165                 170                 175
Arg Lys Gly Lys Asp Ala Arg Ala Leu Met Asn Leu His Asn Asn Arg
            180                 185                 190
Ala Gly Arg Lys Ala Val Lys Arg Phe Leu Lys Gln Glu Cys Lys Cys
        195                 200                 205
His Gly Val Ser Gly Ser Cys Thr Leu Arg Thr Cys Trp Leu Ala Met
    210                 215                 220
Ala Asp Phe Arg Lys Thr Gly Asp Tyr Leu Trp Arg Lys Tyr Asn Gly
225                 230                 235                 240
Ala Ile Gln Val Val Met Asn Gln Asp Gly Thr Gly Phe Thr Val Ala
                245                 250                 255
Asn Glu Arg Phe Lys Lys Pro Thr Lys Asn Asp Leu Val Tyr Phe Glu
            260                 265                 270
Asn Ser Pro Asp Tyr Cys Ile Arg Asp Arg Glu Ala Gly Ser Leu Gly
        275                 280                 285
Thr Ala Gly Arg Val Cys Asn Leu Thr Ser Arg Gly Met Asp Ser Cys
    290                 295                 300
Glu Val Met Cys Cys Gly Arg Gly Tyr Asp Thr Ser His Val Thr Arg
305                 310                 315                 320
Met Thr Lys Cys Gly Cys Lys Phe His Trp Cys Cys Ala Val Arg Cys
                325                 330                 335
Gln Asp Cys Leu Glu Ala Leu Asp Val His Thr Cys Lys Ala Pro Lys
            340                 345                 350
Asn Ala Asp Trp Thr Thr Ala Thr
        355                 360
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 352 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Ala Pro Leu Gly Tyr Leu Leu Val Leu Cys Ser Leu Lys Gln Ala
  1               5                  10                  15
Leu Gly Ser Tyr Pro Ile Trp Trp Ser Leu Ala Val Gly Pro Gln Tyr
            20                  25                  30
Ser Ser Leu Ser Thr Gln Pro Ile Leu Cys Ala Ser Ile Pro Gly Leu
        35                  40                  45
Val Pro Lys Gln Leu Arg Phe Cys Arg Asn Tyr Val Glu Ile Met Pro
    50                  55                  60
Ser Val Ala Glu Gly Val Lys Ala Gly Ile Gln Glu Cys Gln His Gln
65                  70                  75                  80
Phe Arg Gly Arg Arg Trp Asn Cys Thr Thr Val Ser Asn Ser Leu Ala
                85                  90                  95
Ile Phe Gly Pro Val Leu Asp Lys Ala Thr Arg Glu Ser Ala Phe Val
            100                 105                 110
His Ala Ile Ala Ser Ala Gly Val Ala Phe Ala Val Thr Arg Ser Cys
```

```
        115                 120                 125
Ala Glu Gly Ser Ala Ala Ile Cys Gly Cys Ser Ser Arg Leu Gln Gly
    130                 135                 140
Ser Pro Gly Glu Gly Trp Lys Trp Gly Gly Cys Ser Glu Asp Ile Glu
145                 150                 155                 160
Phe Gly Gly Met Val Ser Arg Glu Phe Ala Asp Ala Arg Glu Asn Arg
                165                 170                 175
Pro Asp Ala Arg Ser Ala Met Asn Arg His Asn Asn Glu Ala Gly Arg
            180                 185                 190
Gln Ala Ile Ala Ser His Met His Leu Lys Cys Lys Cys His Gly Leu
        195                 200                 205
Ser Gly Ser Cys Glu Val Lys Thr Cys Trp Trp Ser Gln Pro Asp Phe
    210                 215                 220
Arg Thr Ile Gly Asp Phe Leu Lys Asp Lys Tyr Asp Ser Ala Ser Glu
225                 230                 235                 240
Met Val Val Glu Lys His Arg Glu Ser Arg Gly Trp Val Glu Thr Leu
                245                 250                 255
Arg Pro Arg Tyr Thr Tyr Phe Lys Val Pro Thr Glu Arg Asp Leu Val
            260                 265                 270
Tyr Tyr Glu Ala Ser Pro Asn Phe Cys Glu Pro Asn Pro Glu Thr Gly
        275                 280                 285
Ser Phe Gly Thr Arg Asp Arg Thr Cys Asn Val Ser Ser His Gly Ile
    290                 295                 300
Asp Gly Cys Asp Leu Leu Cys Cys Gly Arg Gly His Asn Ala Arg Thr
305                 310                 315                 320
Glu Arg Arg Arg Glu Lys Cys His Cys Val Phe His Trp Cys Cys Tyr
                325                 330                 335
Val Ser Cys Gln Glu Cys Thr Arg Val Tyr Asp Val His Thr Cys Lys
            340                 345                 350
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 349 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Asn Arg Lys Ala Leu Arg Cys Leu Gly His Leu Phe Leu Ser Leu
  1               5                  10                  15
Gly Met Val Cys Leu Arg Ile Gly Gly Phe Ser Ser Val Val Ala Leu
            20                  25                  30
Gly Ala Thr Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
        35                  40                  45
Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
    50                  55                  60
Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65                  70                  75                  80
Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
                85                  90                  95
Leu Lys Val Gly Ser Arg Asp Gly Ala Phe Thr Tyr Ala Ile Ile Ala
            100                 105                 110
Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr His Gly Asn Leu
        115                 120                 125
Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
    130                 135                 140
Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145                 150                 155                 160
Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
                165                 170                 175
```

```
Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
            180                 185                 190
Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
        195                 200                 205
Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
    210                 215                 220
Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225                 230                 235                 240
Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
                245                 250                 255
Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
            260                 265                 270
Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
        275                 280                 285
Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
    290                 295                 300
Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305                 310                 315                 320
Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
                325                 330                 335
Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
            340                 345
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Gly Val Ser Gly Ser Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro
  1               5                  10                  15
Lys Phe Arg Glu Val Gly His Leu Leu Lys Glu Lys Tyr Asn Ala Ala
            20                  25                  30
Val Gln Val Glu Val Val Arg Ala Ser Arg Leu Arg Gln Pro Thr Phe
        35                  40                  45
Leu Arg Ile Lys Gln Leu Arg Ser Tyr Gln Lys Pro Met Glu Thr Asp
    50                  55                  60
Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Ala Ala
65                  70                  75                  80
Thr Gly Ser Val Gly Thr Gln Gly Arg Ile Cys Asn Arg Thr Ser Pro
                85                  90                  95
Gly Ala Asp Gly Cys Asp Thr Met Cys Cys Gly Arg Gly Tyr Asn Thr
            100                 105                 110
His Gln Tyr Thr Lys Val Trp Gln Cys Asn Cys Lys
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 365 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Gly Ser Ala Met Ser Ser Lys Phe Phe Leu Val Ala Leu Ala
```

```
  1               5                  10                  15
Ile Phe Phe Ser Phe Ala Gln Val Val Ile Glu Ala Asn Ser Trp Trp
            20                  25                  30
Ser Leu Gly Met Asn Asn Pro Val Gln Met Ser Glu Val Tyr Ile Ile
        35                  40                  45
Gly Ala Gln Pro Leu Cys Ser Gln Leu Ala Gly Leu Ser Gln Gly Gln
    50                  55                  60
Lys Lys Leu Cys His Leu Tyr Gln Asp His Met Gln Tyr Ile Gly Glu
65                  70                  75                  80
Gly Ala Lys Thr Gly Ile Lys Glu Cys Gln Tyr Gln Phe Arg His Arg
                85                  90                  95
Arg Trp Asn Cys Ser Thr Val Asp Asn Thr Ser Val Phe Gly Arg Val
            100                 105                 110
Met Gln Ile Gly Ser Arg Glu Thr Ala Phe Thr Tyr Ala Val Ser Ala
        115                 120                 125
Ala Gly Val Val Asn Ala Met Ser Arg Ala Cys Arg Glu Gly Glu Leu
    130                 135                 140
Ser Thr Cys Gly Cys Ser Arg Ala Ala Arg Pro Lys Asp Leu Pro Arg
145                 150                 155                 160
Asp Trp Leu Trp Gly Gly Cys Gly Asp Asn Ile Asp Tyr Gly Tyr Arg
                165                 170                 175
Phe Ala Lys Glu Phe Val Asp Ala Arg Glu Arg Glu Arg Ile His Ala
            180                 185                 190
Lys Gly Ser Tyr Glu Ser Ala Arg Ile Leu Met Asn Leu His Asn Asn
        195                 200                 205
Glu Ala Gly Arg Arg Thr Val Tyr Asn Leu Ala Asp Val Ala Cys Lys
    210                 215                 220
Cys His Gly Val Ser Gly Ser Cys Ser Leu Lys Thr Cys Trp Leu Gln
225                 230                 235                 240
Leu Ala Asp Phe Arg Lys Val Gly Asp Ala Leu Lys Glu Lys Tyr Asp
                245                 250                 255
Ser Ala Ala Ala Met Arg Leu Asn Ser Arg Gly Lys Leu Val Gln Val
            260                 265                 270
Asn Ser Arg Phe Asn Ser Pro Thr Thr Gln Asp Leu Val Tyr Ile Asp
        275                 280                 285
Pro Ser Pro Asp Tyr Cys Val Arg Asn Glu Ser Thr Gly Ser Leu Gly
    290                 295                 300
Thr Gln Gly Arg Leu Cys Asn Lys Thr Ser Glu Gly Met Asp Gly Cys
305                 310                 315                 320
Glu Leu Met Cys Cys Gly Arg Gly Tyr Asp Gln Phe Lys Thr Val Gln
                325                 330                 335
Thr Glu Arg Cys His Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
            340                 345                 350
Lys Lys Cys Thr Glu Ile Val Asp Gln Phe Val Cys Lys
        355                 360                 365
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5607 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGTATGTAT GTATGTATGT ATGTATGTAT ACGTGCGTGC ACCTGTGTGT GCTTGGTGTC
60
AGTGGGGCTC AGACATCACC TGATTCCCTG GAACTGGAGT TACAGGTGGC TATAAGCCAC
120
```

CACTTGGGTG CTGAGAACAG AGTCCGGGCC TCTGGCAGAG CAGTCAGTGC TTTTAGCCAC
180
TGAGCCACTC TCATCCCCCC AATTATGTTC ATCTTGAGTT GGGCAGGTAC GGTGGCGGAA
240
TAGGCCTGTA ATCCCAGCAG TCACTGGACC ATCATGGGTT CTACATATTA AACCTTTATG
300
TTAGGTAGGG TCACACAGCA AGATCCGGTC ACAAAACCAG CAACAACAAA AACCAAAAGG
360
AGCCAGCTTC TTCCCACAAG CATTCTTTCC CTCAGGTCTT CAGCTCCATC TGACAGCTAC
420
TCGGCTGGTG GTCCTATCCT TTCTGAGCCT AGTTGCCAGA GAAACAAGCC CGGTTCATCT
480
TCATGACTAG CACATCTAAT GATAAGCACA GGTTGACTCA AGGTGCCATA GAGTGACACT
540
AGGTACCCAG AGCGACAGAA TGACACCTAT GAGTGCACGT CGTTAATCAC AAACACACAC
600
ACACACACAC ACACACACAC ACACACACAC TCATGCACCC ACCTGCAAAC ACAATTGCAG
660
CCTTCTGGAC GTCTCCTGTC ACAGCCCCAC CTCCTTCCTG ATACACTGCG TTAAGTGGTG
720
ACTGTAACAA AATGACTTCA TGCTCTCCCT GTCCTGAGCC AAATTACACA ATTATTTGGA
780
AAGGGCTCAA AATGTTCTTC GTTAGAAGTT TCTGGATACA CCAATACACA GGAGCGTGCA
840
CCCTCAGAAC ACATGTACAC TTTGACTTAA TCTCACGGGT GACACACCGA CGCTTACACT
900
CCCCCTAGCC CACAGAGGCA AACTGCTGGG CGCTTCTGAG TTTCTCACTG CCACCAGCTC
960
GGTTTGCTCA GCCTACCCCC GCACCCCGCG CCCGGGAATC CCTGACCACA GCTCCACCCA
1020
TGCTCTGTCT CCTTCTTTTC CTTCTCTGTC CAGCCGTCGG GGTTCCTGGG TGAGGAAGTG
1080
TCTCCACGGA GTCGCTGGCT AGAACCACAA CTTTCATCCT GCCATTCAGA ATAGGGAAGA
1140
GAAGAGACCA CAGCGTAGGG GGGACAGAGG AGACGGACTT CGAGAGGACA GCCCCACCGG
1200
CGCGTGTGGG GGAGGCAATC CAGGCTGCAA ACAGGTTGTC CCCAGCGCAT TGTCCCCGCG
1260
CCCCCTGGCG GATGCTGGTC CCCGACGGGC TCCGGACGCG CAGAAGAGTG AGGCCGGCGC
1320
GCGTGGGAGG CCATCCCAAG GGGAGGGGTC GGCGGCCAGT GCAGACCTGG AGGCGGGGCC
1380
ACCAGGCAGG GGGCGGGGGT GAGCCCCGAC GGTTAGCCTG TCAGCTCTTT GCTCAGACCG
1440
GCAAGAGCCA CAGCTTCGCT CGCCACTCAT TGTCTGTGGC CCTGACCAGT GCGCCCTGGT
1500
GCTTTTAGTG CCGCCCGGGC CCGGAGGGGC AGCCTCTTCT CACTGCAGTC AGCGCCGCAA
1560
CTATAAGAGG CCTATAAGAG GCGGTGCCTC CCGCAGTGGC TGCTTCAGCC CAGCAGCCAG
1620
GACAGCGAAC CATGCTGCCT GCGGCCCGCC TCCAGACTTA TTAGAGCCAG CCTGGGAACT
1680
CGCATCACTG CCCTCACCGC TGTGTCCAGT CCCACCGTCG CGGACAGCAA CCACAGTCGT
1740
CAGAACCGCA GCACAGAACC AGCAAGGCCA GGCAGGCCAT GGGGCTCTGG GCGCTGCTGC
1800
CCAGCTGGGT TTCTACTACG TTGCTACTGG CACTGACCGC TCTGCCCGCA GCCCTGGCTG
1860
CCAACAGTAG TGGCCGATGG TGGTAAGTGA GCTAGTACGG GGTCCGCCAC TTGTCCTGGG
1920
GCAAAGAGCC AGGCACGGGC CTTACCCAGC TCCCACGCTG TGGGGATCAC CAACCTACAG
1980

ACCCCCCTCG TGCATTGTGA CTTCACATCC AGGGTGCTCA CACCTAGAAC TAGCTCTGCT
2040
GAAGTGGGGC ACATCATTGG CATGCAGAAG CCCAGATACA CCAGGCTCAG AGACCATTCC
2100
CATTTAATAC GACCCCGTTT CTGCTGAGCA ACAGGTCCCA ACCTCGCTGT GGTGGGTGCT
2160
CAGGTGTCCC TTAGGTCTTG AACCAAAAAA AAAAAAAAAA AAAAAAAAAA ACCAGATATT
2220
AGCTTTGAGG TGAGGGAGTG GAATTCCTAA GTTTTTCAAG GTGGGCAAGG CTGCAGGTGG
2280
GGTTTCTCCT CGGGGGCTGA CTTGAAGAAA GGAAGAGCTA AGGTAGCCAT GCCTTTTCTG
2340
TCCACTCACT AGACTCTGGA GCTCAGGGCC AGGCAAGGAT AGGGTGGTAC AGCCTGTATG
2400
GTTAGGATGC AGGTCCCCTC CCCTGGACTG AACCCTTATG CATCCCGCCA GGGGCATCGT
2460
GAACATAGCC TCCTCCACGA ACCTGTTGAC GGATTCCAAG AGTCTGCAGC TGGTGCTCGA
2520
GCCCAGTCTG CAGCTGCTGA GCCGCAAGCA GCGGCGACTG ATCCGACAGA ACCCGGGGAT
2580
CCTGCACAGC GTGAGTGGAG GGCTCCAGAG CGCTGTGCGA GAGTGCAAAT GGCAATTCCG
2640
AAACCGCCGC TGGAACTGCC CCACTGCTCC GGGGCCCCAC CTCTTCGGCA AGATCGTCAA
2700
CCGAGGTGGG TGCCCAGGAA AGCGACGCTT CCGGGATTAA GGGAAAAGCA GGGTCATCTC
2760
CAGGGCATAG GCGGGCGAAG GCAGGGAAGA CATCCCAGGG TTATATGTGA TCAAACTGAG
2820
AATCGCCTGG TGCCGGCAGT TACCGTAGGT CAGCACCAGA TTCTTTCTAG CCTTGCGTTG
2880
TGAGCATGAT CTTTAACGTT GCTGGCCACT GGCCCACAGA AAGGGAATTC CGGATCGTGG
2940
GCGCTGGGCG ACAGCTGTTT TTCCCTAGCC TTCCTCAAAG GTACCTGGGA AGCTGATCTC
3000
TGAGGGCTAG CTAGGGTTGT GCTTCGCACC CAGCAAAGTT TGCACTGCCA ATACTAGTAG
3060
CGATCTTGGC TATGCAGATT TGTTCTACTT GGGAATCTCC CCTTGGAGCT GCTCTGCTAG
3120
GGCTCTGGAG TCTCAGTAAA GCTTAGAGAG GAGGGCATTC CATGCTTCGC ACACATGACT
3180
CCAAGGATGT TGGACTGTAG GGTACCAAGT CTTCCAAACA GGGTGCTGAG TTGGCCCCAC
3240
GCCTTCTCTC AACTGATGCG GGGTCGCTTC ACCCACAGGC TGCCGAGAAA CAGCGTTCAT
3300
CTTCGCAATC ACCTCCGCCG GGGTCACACA TTCCGTGGCG CGCTCCTGCT CCGAAGGCTC
3360
CATCGAGTCC TGCACCTGCG ACTACCGGCG GCGCGGCCCT GGGGGCCCCG ACTGGCACTG
3420
GGGGGGCTGC AGTGACAACA TCGATTTTGG TCGCCTCTTT GGCCGAGAGT TCGTGGACTC
3480
CGGGGAGAAG GGGCGGGACC TACGCTTCCT CATGAACCTT CACAACAACG AGGCAGGGCG
3540
AACGGTACGT CGGTGTGTCC GGAACCAATG GCAGGGGAGA TGTAAGACAG GTGCACGGGG
3600
ACAGAGGCAC AGGGAGGGGC TTCCCGAGAG AGTGGGACTC TAGGAGGGAA GACAGAGAAG
3660
AGGTGGTGGT TGAGGGCAAA GAGGTTCCTG AGCTGATGAC AGAACAGAAG AGATTAGCAG
3720
GCTATCAACA CGTGGGATGT ATTGAGATGG CTCCATGGCA CACTTTTGAA AGATAAAAGT
3780
GACTTGCTGG CGTGGAGCAG AGTCTGGCCG AATGTCCCTA TCTCAGCGGG CCATTTTGCA
3840

CTTCCTCTCT CCCGAGCTTA GTCACACCTG GACCTTGGCT GAAGTTTCCA CAGCATCGAC
3900
GTGACCCGGG TGGGGTGGGG GTGGGGAAGT ATGGGTGGTG GTTCGTGGGA TGTTGGCTTT
3960
GACCTTTTCT TCCCTCCTCC CCTCGTCCCC TCCTCCCCCA GACCGTGTTC TCTGAGATGC
4020
GCCAAGAGTG CAAATGCCAC GGGATGTCCG GCTCCTGCAC GGTGCGCACG TGTTGGATGC
4080
GGCTGCCCAC GCTGCGCGCT GTGGGCGACG TGCTGCGCGA CCGCTTCGAC GGCGCCTCCC
4140
GCGTCCTTTA CGGCAACCGA GGCAGCAACC GCGCCTCGCG GGCGGAGCTG CTGCGCCTGG
4200
AGCCCGAAGA CCCCGCGCAC AAGCCTCCCT CCCCTCACGA CCTCGTCTAC TTCGAGAAAT
4260
CGCCCAACTT CTGCACGTAC AGTGGCCGCC TGGGCACAGC TGGCACAGCT GGACGAGCTT
4320
GCAACAGCTC GTCTCCCGCG CTGGACGGCT GTGAGCTGCT GTGCTGTGGC CGAGGCCACC
4380
GCACGCGCAC GCAGCGCGTC ACGGAGCGCT GCAACTGCAC CTTCCACTGG TGCTGCCACG
4440
TCAGCTGCCG CAACTGCACG CACACGCGCG TTCTGCACGA GTGTCTATGA GGTGCCGCGC
4500
CTCCGGGAAC GGGAACGCTC TCTTCCAGTT CTCAGACACA CTCGCTGGTC CTGATGTTTG
4560
CCCACCCTAC CGCGTCCAGC CACAGTCCCA GGGTTCATAG CGATCCATCT CTCCCACCTC
4620
CTACCTGGGG ACTCCTGAAA CCACTTGCCT GAGTCGGCTC GAACCCTTTT GCCATCCTGA
4680
GGGCCCTGAC CCAGCCTACC TCCCTCCCTC TTTGAGGGAG ACTCCTTTTG CACTGCCCCC
4740
CAATTTGGCC AGAGGGTGAG AGAAAGATTC TTCTTCTGGG GTGGGGGTGG GGAGGTCAAC
4800
TCTTGAAGGT GTTGCGGTTC CTGATGTATT TTGCGCTGTG ACCTCTTTGG GTATTATCAC
4860
CTTTCCTTGT CTCTCGGGTC CCTATAGGTC CCTTGAGTTC TCTAACCAGC ACCTCTGGGC
4920
TTCAAGGCCT TTCCCCTCCC ACCTGTAGCT GAAGAGTTTC CGAGTTGAAA GGGCACGGAA
4980
AGCTAAGTGG GAAAGGAGGT TGCTGGACCC AGCAGCAAAA CCCTACATTC TCCTTGTCTC
5040
TGCCTCGGAG CCATTGAACA GCTGTGAACC ATGCCTCCCT CAGCCTCCTC CCACCCCTTC
5100
CTGTCCTGCC TCCTCATCAC TGTGTAAATA ATTTGCACCG AAATGTGGCC GCAGAGCCAC
5160
GCGTTCGGTT ATGTAAATAA AACTATTTAT TGTGCTGGGT TCCAGCCTGG GTTGCAGAGA
5220
CCACCCTCAC CCCACCTCAC TGCTCCTCTG TTCTGCTCGC CAGTCCTTTT GTTATCCGAC
5280
CTTTTTTCTC TTTTACCCAG CTTCTCATAG GCGCCCTTGC CCACCGGATC AGTATTTCCT
5340
TCCACTGTAG CTATTAGTGG CTCCTCGCCC CCACCAATGT AGTATCTTCC TCTGAGGAAT
5400
AAAATATCTA TTTTTATCAA CGACTCTGGT CCTTGAATCC AGAACACAGC ATGGCTTCCA
5460
ACGTCCTCTT CCCTTCCAAT GGACTTGCTT CTCTTCTCAT AGCCAAACAA AAGAGATAGA
5520
GTTGTTGAAG ATCTCTTTTC CAGGGCCTGA GCAAGGACCC TGAGATCCTG ACCCTTGGAT
5580
GACCCTAAAT GAGACCAACT AGGGATC
5607

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2301 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCAGAGCGG ACGGGCGCGC GGGAGGCGCG CAGAGCTTTC GGGCTGCAGG CGCTCGCTGC
60
CGCTGGGGAA TTGGGCTGTG GGCGAGGCGG TCCGGGCTGG CCTTTATCGC TCGCTGGGCC
120
CATCGTTTGA AACTTTATCA GCGAGTCGCC ACTCGTCGCA GGACCGAGCG GGGGGCGGGG
180
GCGCGGCGAG GCGGCGGCCG TGACGAGGCG CTCCCGGAGC TGAGCGCTTC TGCTCTGGGC
240
ACGCATGGCG CCCGCACACG GAGTCTGACC TGATGCAGAC GCAAGGGGGT TAATATGAAC
300
GCCCCTCTCG GTGGAATCTG GCTCTGGCTC CCTCTGCTCT TGACCTGGCT CACCCCCGAG
360
GTCAACTCTT CATGGTGGTA CATGAGAGCT ACAGGTGGCT CCTCCAGGGT GATGTGCGAT
420
AATGTGCCAG GCCTGGTGAG CAGCCAGCGG CAGCTGTGTC ACCGACATCC AGATGTGATG
480
CGTGCCATTA GCCAGGGCGT GGCCGAGTGG ACAGCAGAAT GCCAGCACCA GTTCCGCCAG
540
CACCGCTGGA ATTGCAACAC CCTGGACAGG GATCACAGCC TTTTTGGCAG GGTCCTACTC
600
CGAAGTAGTC GGGAATCTGC CTTTGTTTAT GCCATCTCCT CAGCTGGAGT TGTATTTGCC
660
ATCACCAGGG CCTGTAGCCA AGGAGAAGTA AAATCCTGTT CCTGTGATCC AAAGAAGATG
720
GGAAGCGCCA AGGACAGCAA AGGCATTTTT GATTGGGGTG GCTGCAGTGA TAACATTGAC
780
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840
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900
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960
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1020
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1080
CCAACGAAAA ATGACCTCGT GTATTTTGAG AATTCTCCAG ACTACTGTAT CAGGGACCGA
1140
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1200
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1260
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1380
CAGCAGGCGT CACCATCCAC CTTCCCTTCT ACAAGGACTC CATTGGATCT GCAAGAACAC
1440
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1500
GAAGCCCCCT CTTCCTCCCT GGGGGCCCCA GGATGGGGGG CCACACGCTG CACCTAAAGC
1560

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1620
TTTGGAAATA GCATGACAGG CTGTTCAGCC GGGAGGGTGG TGGGCCCAGA CCACTGTCTC
1680
CACCCACCTT GACGTTTCTT CTTTCTAGAG CAGTTGGCCA AGCAGAAAAA AAAGTGTCTC
1740
AAAGGAGCTT TCTCAATGTC TTCCCACAAA TGGTCCCAAT TAAGAAATTC CATACTTCTC
1800
TCAGATGGAA CAGTAAAGAA AGCAGAATCA ACTGCCCCTG ACTTAACTTT AACTTTTGAA
1860
AAGACCAAGA CTTTTGTCTG TACAAGTGGT TTTACAGCTA CCACCCTTAG GGTAATTGGT
1920
AATTACCTGG AGAAGAATGG CTTTCAATAC CCTTTTAAGT TTAAAATGTG TATTTTTCAA
1980
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2040
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2100
GTCCCCTTGA TATAAAAAAT CTTTAGGGAA TGAGAGTTCC TTCTCACTTA GAATCTGAAG
2160
GGAATTAAAA AGAAGATGAA TGGTCTGGCA ATATTCTGTA ACTATTGGGT GAATATGGTG
2220
GAAAATAATT TAGTGGATGG AATATCAGAA GTATATCTGT ACAGATCAAG AAAAAAAGGA
2280
AGAATAAAAT TCCTATATCA T
2301

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2814 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCATGT CTTACGGTCA AGGCAGAGGG CCCAGCGCCA CTGCAGCCGC GCCACCTCCC
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AGGGCCGGGC CAGCCCAGGC GTCCGCGCTC TCGGGGTGGA CTCCCCCCGC TGCGCGCTCA
120
AGCCGGCGAT GGCTCCTCTC GGATACCTCT TAGTGCTCTG CAGCCTGAAG CAGGCTCTGG
180
GCAGCTACCC GATCTGGTGG TCCTTGGCTG TGGGACCCCA GTACTCCTCT CTGAGCACTC
240
AGCCCATTCT CTGTGCCAGC ATCCCAGGCC TGGTACCGAA GCAGCTGCGC TTCTGCAGGA
300
ACTACGTGGA GATCATGCCC AGCGTGGCTG AGGGTGTCAA AGCGGGCATC CAGGAGTGCC
360
AGCACCAGTT CCGAGGCCGG CGTTGGAACT GCACCACCGT CAGCAACAGC CTGGCCATCT
420
TTGGCCCTGT TCTGGACAAA GCCACCCGGG AGTCAGCCTT TGTCCATGCC ATCGCCTCCG
480
CTGGAGTAGC TTTCGCAGTG ACACGCTCCT GTGCAGAGGG ATCAGCTGCT ATCTGTGGGT
540
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600
ACATTGAATT TGGAGGAATG GTCTCTCGGG AGTTTGCCGA TGCCAGGGAG AACCGGCCGG
660
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840
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900
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960
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1080
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1140
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1200
ACGGGAGCAG GGTTCATTCC GAGGGGCAAG GTTCCTACCT GGGGGCGGGG TTCCTACTTG
1260
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1320
GTCTCATACC TAAGGACCCG GTTTCTGCCT TCAGCCTGGG CTCCTATTTG GGATCTGGGT
1380
TCCTTTTTAG GGGAGAAGCT CCTGTCTGGG ATACGGGTTT CTGCCCGAGG GTGGGGCTCC
1440
ACTTGGGGAT GGAATTCCAA TTTGGGCCGG AAGTCCTACC TCAATGGCTT GGACTCCTCT
1500
CTTGACCCGA CAGGGCTCAA ATGGAGACAG GTAAGCTACT CCCTCAACTA GGTGGGGTTC
1560
GTGCGGATGG GTGGGAGGGG AGAGATTAGG GTCCCTCCTC CCAGAGGCAC TGCTCTATCT
1620
AGATACATGA GAGGGTGCTT CAGGGTGGGC CCTATTTGGG CTTGAGGATC CCGTGGGGGC
1680
GGGGCTTCAC CCCGACTGGG TGGAACTTTT GGAGACCCCC TTCCACTGGG GCAAGGCTTC
1740
ACTGAAGACT CATGGGATGG AGCTCCACGG AAGGAGGAGT TCCTGAGCGA GCCTGGGCTC
1800
TGAGCAGGCC ATCCAGCTCC CATCTGGCCC CTTTCCAGTC CTGGTGTAAG GTTCAACCTG
1860
CAAGCCTCAT CTGCGCAGAG CAGGATCTCC TGGCAGAATG AGGCATGGAG AAGAACTCAG
1920
GGGTGATACC AAGACCTAAC AAACCCCGTG CCTGGGTACC TCTTTTAAAG CTCTGCACCC
1980
CTTCTTCAAG GGCTTTCCTA GTCTCCTTGG CAGAGCTTTC CTGAGGAAGA TTTGCAGTCC
2040
CCCAGAGTTC AAGTGAACAC CCATAGAACA GAACAGACTC TATCCTGAGT AGAGAGGGTT
2100
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2160
AGCCTGCATC CGCTCTGACA CTTAATACTC AGATCTCCCG GGAAACCCAG CTCATCCGGT
2220
CCGTGATGTC CATGCCCCAA ATGCCTCAGA GATGTTGCCT CACTTTGAGT TGTATGAACT
2280
TCGGAGACAT GGGGACACAG TCAAGCCGCA GAGCCAGGGT TGTTTCAGGA CCCATCTGAT
2340
TCCCCAGAGC CTGCTGTTGA GGCAATGGTC ACCAGATCCG TTGGCCACCA CCCTGTCCCG
2400
AGCTTCTCTA GTGTCTGTCT GGCCTGGAAG TGAGGTGCTA CATACAGCCC ATCTGCCACA
2460
AGAGCTTCCT GATTGGTACC ACTGTGAACC GTCCCTCCCC CTCCAGACAG GGGAGGGGAT
2520
GTGGCCATAC AGGAGTGTGC CCGGAGAGCG CGGAAAGAGG AAGAGAGGCT GCACACGCGT
2580

GGTGACTGAC TGTCTTCTGC CTGGAACTTT GCGTTCGCGC TTGTAACTTT ATTTTCAATG
2640
CTGCTATATC CACCCACCAC TGGATTTAGA CAAAAGTGAT TTTCTTTTTT TTTTTTTCTT
2700
TTCTTTCTAT GAAAGAAATT ATTTTAGTTT ATAGTATGTT TGTTTCAAAT AATGGGGAAA
2760
GTAAAAAGAG AGAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAA
2814

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Cys Lys Cys His Gly Leu Ser Gly Ser Cys Glu Val Lys Thr Cys Trp
1 5 10 15
Trp Ser Gln Pro Asp Phe Arg Ala Ile Gly Asp Phe Leu Lys Asp Lys
20 25 30
Tyr Asp Ser Ala Ser Glu Met Val Val Glu Lys His Arg Glu Ser Arg
35 40 45
Gly Trp Val Glu Thr Leu Arg Pro Arg Tyr Thr Tyr Phe Lys Val Pro
50 55 60
Thr Glu Arg Asp Leu Val Tyr Tyr Glu Ala Ser Pro Asn Phe Cys Glu
65 70 75 80
Pro Asn Pro Glu Thr Gly Ser Phe Gly Thr Arg Asp Arg Thr Cys Ans
85 90 95
Val Ser Ser His Gly Ile Asp Gly Cys Asp Leu Leu Cys Cys Gly Arg
100 105 110
Gly His Asn Ala Arg Ala Glu Arg Arg Arg Glu Lys Cys Arg Cys Val
115 120 125
Phe His Trp Cys Cys
130

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGTAAGTGCC ACGGGCTGTC GGGCAGCTGC GAGGTGAAGA CATGCTGGTG GTCGCAACCC
60
GACTTCCGCG CCATCGGTGA CTTCCTCAAG GACAAGTACG ACAGCGCCTC GGAGATGGTG
120
GTGGAGAAGC ACCGGGAGTC CCGCGGCTGG GTGGAGACCC TGCGGCCGCG CTACACCTAC
180
TTCAAGGTGC CCACGGAGCG CGACCTGGTC TACTACGAGG CCTCGCCCAA CTTCTGCGAG
240
CCCAACCCTG AGACGGGCTC CTTCGGCACG CGCGACCGCA CCTGCAACGT CAGCTCGCAC
300

GGCATCGACG GCTGCGACCT GCTGTGCTGC GGCCGCGGCC ACAACGCGCG AGCGGAGCGG
360
CGCCGGGAGA AGTGCCGCTG CGTGTTTCAC TGGTGCTGT
399

What is claimed is:

1. An enriched population of mammalian neural precursor cells committed to a cell fate, said cells being characterized in that they exhibit a stem cell phenotype in the presence of a Wnt polypeptide but not in the absence of said Wnt polypeptide.

2. An enriched population of mammalian dopaminergic neuron precursor cells, said cells being characaterized in that they exhibit a stem cell phenotype in the presence of a Wnt polypeptide and differentiate into dopaminergic neurons in the absence of said Wnt polypeptide.

3. The population of claim 2, wherein said Wnt polypeptide is a Wnt-1 class polypeptide.

4. The population of claim 3, wherein said Wnt polypeptide is selected from the group consisting of Wnt-1, Wnt-2, Wnt-3a, Wnt-7a, and Wnt-7b.

5. The population of claim 4, wherein said Wnt polypeptide is Wnt-1.

6. The population of claim 5, wherein said Wnt-1 polypeptide has a sequence that is at least 80% identical to SEQ ID NO: (human Wnt-1).

7. The population of claim 2, wherein said cells are human cells.

8. The population of claim 7, wherein said cells are fetal human cells.

9. The population of claim 2, wherein said cells are porcine cells.

10. An enriched population of mammalian dorsal hindbrain precursor cells, said cells being characterized in that they exhibit a stem cell phenotype in the presence of both a Wnt-1 polypeptide and a Wnt-3a polypeptide but not in the absence of said Wnt-1 polypeptide and said Wnt-3a polypeptide.

11. An enriched population of mammalian hippocampal neuron precursor cells, said cells being characterized in that they exhibit a stem cell phenotype in the presence of a Wnt-3a polypeptide and differentiate into hippocampal neurons in the absence of said Wnt-3a polypeptide..

12. The population of claim 11, wherein said Wnt-3a polypeptide has a sequence that is at least 80% identical to SEQ ID NO: (mouse Wnt-3a).

13. The population of claim 11, wherein said cells are human cells.

14. A method of treating a heterogeneous population of neural cell precursor cells to enrich for dorsal neural precursor cells, comprising culturing said population with Wnt polypeptide, wherein said dorsal neural precursor cells selectively proliferate in the presence of said Wnt polypeptide.

15. A method of stimulating cell proliferation of a dorsal neural precursor cell comprising contacting said cell with a Wnt-1 polypeptide or a Wnt-3a polypeptide.

16. The method of claim 15, wherein said cell is contacted with both a Wnt-1 polypeptide and a Wnt-3a polypeptide.

17. A method of inducing neuronal regeneration in an adult mammal suffering from a neurodegenerative disorder, comprising transplanting into said mammal an enriched population of dorsal neural precursor cells.

18. The method of claim 17, wherein said disorder is Parkinson's Disease, Amyotrophic Lateral Sclerosis, Diffuse Lewy Body Disease, Cortical-basal Ganglionic Degeneration, Hallervorden-Spatz Disease, or Myoclonic Epilepsy.

19. The method of claim 17, further comprising administering to said mammal a Wnt polypeptide or Wnt agonist.

20. A method of treating Parkinson's disease, comprising transplanting into the brain of a patient an enriched population of dopaminergic neuron precursor cells.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08716

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 5/06, 5/08
US CL :435/325, 368, 377
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 368, 377

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE
search terms: neural, precursor#, progenitor, stem, cell#, human, porcine, wnt#, dorsal, hippocam##, hindbrain, dopamin?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,411,883 A (BOSS et al) 02 May 1995, columns 3, 7, 11-13, 17 and 19-20.	1-9 --- 10-13
X --- A	US 5,589,376 A (ANDERSON et al) 31 December 1996, columns 3-4, 8-9, 11, 13-14 and 16-17.	1 --- 2-13
X	MOYER et al. Culture, Expansion, and Transplantation of Human Fetal Neural Progenitor Cells. Transplantation Proceedings. June 1997, Vol. 29, No. 4, pages 2040-2041, see entire document.	1-8, 10-13
X	US 5,656,481 A (BAETGE et al) 12 August 1997, column 30, lines 46-57.	1, 11-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 JULY 1998	Date of mailing of the international search report 31 AUG 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROBERT C. HAYES, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08716

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-13

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08716

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-13, drawn to a population of mammalian neural precursor cells committed to a cell fate.

Group II, claim(s) 14-16, drawn to a method of stimulating proliferation of a heterogenous population of neural cell precursor cells to enrich for dorsal neural cells.

Group III, claim(s) 17-18 and 20, drawn to a method of inducing neuronal regeneration in an adult mammal comprising transplanting dorsal neural precursor cells.

Group IV, claim(s) 19, drawn to a method of inducing neuronal regeneration in an adult mammal comprising administering a Wnt polypeptide or Wnt agonist.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is directed to a population of mammalian neural precursor cells, which is the first product. However, because Boss et al teach an enriched population of porcine or human neuron progenitor cells (i.e., mammalian neural precursor cells), no special technical feature exists for Group I as defined by PCT RULE 13.2, because it does not define a contribution over the prior art. The technical features of Groups II-IV are drawn to methods having different goals, method steps and starting materials, which do not share the same or a corresponding technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application. Because the technical feature of Group I is not a special technical feature, and because the technical features of the Group II-IV inventions are not present in the Group I claims, unity of invention is lacking.

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DEV2327

Wnt-4 is a mesenchymal signal for epith lial transformation of metanephric mesenchyme in the developing kidney

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SUMMARY

Development of the mammalian kidney is initiated by ingrowth of the ureteric bud into the metanephric blastema. In response to signal(s) from the ureter, mesenchymal cells condense, aggregate into pretubular clusters, and undergo epithelialisation to form simple epithelial tubules. Subsequent morphogenesis and differentiation of the tubular epithelium lead to the establishment of a functional nephron.

Here we demonstrate that Wnt-4, a secreted glycoprotein which is required for tubule formation, is sufficient to trigger tubulogenesis in isolated metanephric mesenchyme, whereas Wnt-11 which is expressed in the tip of the growing ureter is not. Wnt-4 signaling depends on cell contact and sulphated glycosaminoglycans and is only required for triggering tubulogenesis but not for later events. The Wnt-4 signal can be replaced by other members of the *Wnt* gene family including Wnt-1, Wnt-3a, Wnt-7a and Wnt-7b. Further, dorsal spinal cord, which has been thought to mimic ureteric signaling in tubule induction induces *Wnt-4* mutant as well as wild-type mesenchyme suggesting that spinal cord derived signal(s) most likely act by mimicking the normal mesenchymal action of Wnt-4. These results lend additional support to the notion that Wnt-4 is a key auto-regulator of the mesenchymal to epithelial transformation that underpins nephrogenesis adding another level of complexity in the hierarchy of molecular events mediating tubulogenesis.

Key words: Wnt, Wnt-4, Induction, Tubulogenesis, Kidney development, Metanephros

INTRODUCTION

The development of vertebrate organs requires intricate cell and tissue interactions to assure the concerted program of cell growth, differentiation and morphogenesis. The mammalian kidney, mainly of mouse and rat, has long been studied as a model system to reveal both the embryological principles and more recently the molecular control of vertebrate organ formation (for reviews see Saxen, 1987; Lechner and Dressler, 1997; Vainio and Muller, 1997).

Mouse renal development is characterised by the continuous interaction of epithelial and mesenchymal compartments both of which derive from the intermediate mesenchyme. These are the nephric duct and its derivative the ureter, and the nephrogenic mesenchyme which lies adjacent to these ducts. As a consequence of these interactions three embryonic kidneys are laid down from anterior to posterior in time and space. While the initial organ, the pronephros is only a very transient structure established at 8-8.5 days post coitum (d p.c.), the mesonephros extends by posterior elongation of the nephric duct and subsequent tubule induction in the adjacent mesonephrogenic mesenchyme between 9 and 11 d p.c. Although forming elaborate tubules, the mesonephros of the male never becomes a functional organ but contributes to the ductal network of the rete testis.

Metanephric development is initiated when a bud emerges from the nephric duct at the level of the hind limbs around 10.5 d p.c. The ureteric duct subsequently invades the metanephric blastema which lies at the posterior end of the intermediate mesoderm.

In a process repeated many times, mesenchymal cells condense around the tip of the ureter, i.e. bud, aggregate, epithelialise and undergo morphogenetic movements and cellular differentiation programs to form a major part of the nephron the functional unit of the vertebrate kidney. The ureter continues to grow and to branch forming the collecting duct system of the mature organ. 7-10 days post partum nephron formation ceases most likely as the mesenchymal stem cells in the periphery of the kidney are exhausted.

In order to achieve the complex architecture of the mature kidney, the morphogenetic and cellular differentiation programs of both the nephric duct and the mesenchymal derivatives have to be highly integrated making it very likely that multiple signaling systems between and within the two compartments are operative.

Classical kidney organ culture experiments have primarily

focused on the signals exchanged between mesenchyme and ureter upon their initial contact. Separation and recombination experiments have shown that isolated metanephric mesenchyme undergoes apoptosis unless provided with a permissive stimulus which leads to epithelial tubule formation (Grobstein, 1953; Saxen, 1987). Similarly, ureter tissue degenerates upon separation from metanephric mesenchyme, and undergoes limited or altered branching morphogenesis when recombined with heterologous mesenchymal tissues suggesting that metanephric mesenchyme secretes signals essential for ureter survival and correct branching morphogenesis (Saxen, 1987; Kispert et al., 1996; Sainio et al., 1997).

A search for heterologous tissues that may be a more convenient source of a factor capable of replicating ureteric signaling has identified the dorsal spinal cord as a potent inducer of tubulogenesis. Indeed, most of our understanding of cell interactions in kidney development comes from the application of spinal cord derived signals to isolated metanephric mesenchyme in culture. These studies have demonstrated that induction appears to be cell contact dependent, requires approximately 24 hours of contact between spinal cord and mesenchyme, can be blocked by metabolic inhibitors, and cannot be transferred from induced to uninduced mesenchyme (homeogenetic induction) (Grobstein, 1953; Saxen, 1987, and references therein).

It is important to note that while this assay has generally been thought to provide information about ureteric-like inductive signals there is no evidence that this is the case. For example, induction of tubules may require the action of several signals, some from the ureter others from the mesenchyme. Supplying any of these may be sufficient to trigger tubule formation.

While recently progress has been made in identifying mesenchymally derived signaling molecules required for ureter proliferation and branching morphogenesis (see Sariola and Sainio, 1997, for review) the nature of ureteric signals has remained elusive. Several growth factors have been discussed as potential inducers (Hammerman, 1995) but none of them has conclusively been shown to be required and sufficient for tubule induction. A combination of FGF2 and a pituitary extract can induce tubulogenesis, suggesting that tubule induction is a multi-step process mediated by soluble and possibly insoluble factors (Perantoni, 1991; Perantoni et al., 1995).

Several findings have implicated members of the Wnt family of secreted glycoproteins in signaling processes operating during metanephric development. *Wnt-11* is expressed in the tips of the growing ureter where tubule inducing activity is thought to arise (Kispert et al., 1996). In contrast, *Wnt-4* is expressed in pretubular mesenchyme cells shortly before they aggregate and transform to simple epithelial tubules. Loss of function studies indicate that *Wnt-4* is required for tubule formation (Stark et al., 1994). Finally, *Wnt-7b* is expressed somewhat later in the collecting duct epithelium which derives from the ureteric duct (Kispert et al., 1996). NIH3T3 cells stably expressing *Wnt-1*, which is not expressed in the kidney but is expressed in the dorsal spinal cord, are able to induce tubulogenesis in isolated rat metanephric mesenchyme (Herzlinger et al., 1994). This suggests that a member of the Wnt family may normally participate in tubule induction. Thus, induction in culture by the spinal cord might mimic ureteric signaling by Wnt-11 *or* mesenchymal signaling by Wnt-4.

Here we describe a number of experiments in which we investigate the role of *Wnt-11*, *Wnt-4* and a number of other family members in tubule induction. From these results we conclude that Wnt-4, but not Wnt-11, is able to induce tubule formation, and that spinal cord mediated tubulogenesis may reflect the normal mesenchymal function of Wnt-4 rather than that of a ureteric bud derived signal.

MATERIALS AND METHODS

Mice

Wnt-4 heterozygotes were derived and genotyped as described previously (Stark et al., 1994). Embryos for kidney dissections were derived from matings of Swiss Webster (SW) wild-type animals or *Wnt-4* heterozygotes. For timed pregnancies plugs were checked in the morning after mating, noon was taken as 0.5 days post coitum (d p.c.).

Cell lines

Cell lines stably expressing various *Wnt* genes or *lacZ* were prepared essentially as described (Pear et al., 1993). Full-length cDNAs encoding *Wnt-1* (van Ooyen and Nusse, 1984), *Wnt-3a* (Roelink and Nusse, 1991), *Wnt-4*, *Wnt-5a*, *Wnt-7a*, *Wnt-7b* (Gavin et al., 1990), *Wnt-11* (Kispert et al., 1996) and *lacZ* were cloned into the retroviral expression vector pLNCX which confers expression of foreign genes under the control of the CMV promotor (Miller and Rosman, 1989). Bosc23 packaging cells were transfected with recombinant DNA constructs. Viral supernatants were collected 48-72 hours later and used to infect NIH3T3 cells. After 10 days of selection in G418, pools of cells were used for recombination experiments. 50,000 cells were plated in 50 μl of medium on polycarbonate filter and grown for 18-24 hours at 37°C in 5% CO_2.

Organ culture techniques

Metanephric kidneys from SW or *Wnt-4* intercrosses were dissected in PBS. Metanephric mesenchyme was dissected manually from the ureter (bud stage, 10.75 d p.c., to early T stage, 11.5 d p.c.), following a 2 minute incubation in 3% pancreatin/trypsin (Gibco-BRL) in Tyrode's solution. In recombination experiments with wild-type mesenchymes samples were pooled before being distributed to individual experiments. In experiments with *Wnt-4* mutant embryos metanephric mesenchyme from each kidney of the embryo was kept separate. The remainder of an embryo was used for genotyping by Southern analysis. In recombination experiments with dorsal spinal cord metanephric mesenchyme from two kidneys was surrounded by two dissected pieces of dorsal spinal cord from the same embryo on a 1 μm polycarbonate filter (Costar). For direct recombination experiments with *Wnt* expressing cells two mesenchymes were placed on top of modified NIH3T3 cells. For transfilter experiments 50,000 cells in 50 μl medium were seeded on a 1 μm filter 18-24 hours prior to the recombination. Cells were then covered with a 1 μm filter and two mesenchymes placed on this filter. Filters (4-6 mm in size) were supported by stainless steel grids on the surface of the culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1× penicillin/streptomycin). Medium was changed every 2 days. For glycosaminoglycan dependence of induction the medium was supplemented with 30 mM $NaClO_3$ after 0 hours, 24 hours and 48 hours, respectively. In experiments concerning pore size dependence of induction the pore size of the upper filter in the transfilter set-up was varied from 0.05 μm, 0.1 μm, 0.4 μm, 0.8 μm to 1 μm. The number of cultures performed are indicated in the text. For marker experiments at least 6 specimens were processed.

For in situ hybridisation analysis filters were submerged in cold methanol for 10 seconds and then fixed in 4% paraformaldehyde in PBS overnight prior to stepwise transfer into methanol and storage at -20°C. For histological analysis filters were fixed in Bouin's solution and stored in 70% ethanol at 4°C.

In situ hybridisation analysis

In situ hybridisation analyses on whole mount cultures were performed as described (Kispert et al., 1996). Full-length cDNAs for *WT-1* (Pritchard-Jones et al., 1990), *Pax-2* (Dressler et al., 1990), *Pax-8* (Plachov et al., 1990), *Wnt-4* (Gavin et al., 1990) and *E-cadherin* (Ringwald et al., 1987) were labeled with digoxigenin for whole mount detection.

Histological analysis

Samples were dehydrated, embedded in wax and sectioned at 5 μm. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.

Documentation

Brightfield images of cultures and marker stainings were taken with a binocular on Kodak 64T slide film. Histological sections were photographed on the same film on a Leitz Axiophot. Slides were scanned and figures composed in Adobe Photoshop 4.0.

RESULTS

Spinal cord mimics a mesenchymal signal for tubule induction

The identification of *Wnt-4* as a mesenchymal signal essential for tubule formation provides an excellent new tool for readdressing the role of spinal cord explants as heterologous inducers of kidney tubulogenesis. Clearly, if the spinal cord mimics a ureteric signal upstream of *Wnt-4*, this signal would not rescue the mesenchymal requirement for *Wnt-4* in tubulogenesis. To test this possibility, isolated metanephric mesenchyme from individual embryos derived from intercrosses between mice heterozygous for a likely null allele of *Wnt-4* were cultured on a polycarbonate filter in direct contact with dorsal spinal cord from the same embryo. In the absence of spinal cord, all mesenchyme cultures rapidly degenerated as expected. Surprisingly, when cultured in the presence of spinal cord, mesenchyme from *Wnt-4* mutant embryos developed as well as that of wild-type or heterozygous siblings (Table 1). After 48 hours induction was visible as bright round zones of condensing mesenchyme. After 96 hours the zones of condensing mesenchyme had undergone epithelialisation to form complex tubules. At 144 hours epithelial tubular structures and glomeruli indicated that full differentiation of induced tubules occurred in all recombinants (Fig. 1). Thus, the induction of tubulogenesis in *Wnt-4* mutant mesenchyme indicates that spinal cord signaling acts by either mimicking the action of Wnt-4 itself, or a factor downstream of *Wnt-4*. Further, although *Wnt-4* is expressed in the spinal cord (Parr et al., 1993), the observation that spinal cord from *Wnt-4* mutants is capable of induction indicates that *Wnt-4* expression in the spinal cord is not essential for this process. This leaves open the involvement of other *Wnts* expressed in this tissue.

Table 1. Induction of tubulogenesis in *Wnt-4/Wnt-4* mutant metanephric mesenchyme by dorsal spinal cord

Exp. #	# Recombinants	#Induced/#Total		
		+/+	*Wnt-4/+*	*Wnt-4/Wnt-4*
1	8	2/2	5/5	1/1
2	7	1/1	3/3	3/3
3	7	3/3	3/3	1/1
4	5	1/1	3/3	1/1
5	9	3/3	4/4	2/2
6	11	7/7	4/4	–
7	11	3/3	4/4	4/4
Total	58	20/20	26/26	12/12

Isolated metanephric mesenchyme was recombined with dorsal spinal cord from the same embryo on a nucleopore filter. Induction was monitored by bright field microscopy. Embryos of a total of seven litters were analysed in this way.

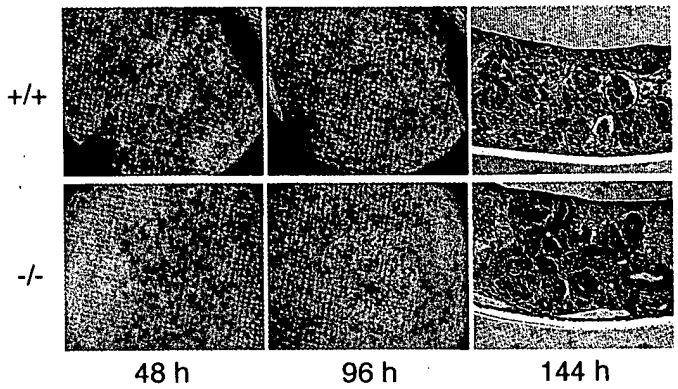

Fig. 1. Induction of tubulogenesis in wild-type and *Wnt-4* mutant metanephric mesenchyme by dorsal spinal cord. Isolated metanephric mesenchyme and dorsal spinal cord from the same 11.5 day embryo were recombined on a nucleopore filter. After 48 hours and 96 hours cultures were monitored as whole mounts using bright field microscopy, after 144 hours as histological sections. Induction of tubulogenesis in wild-type and *Wnt-4/Wnt-4* mutant metanephric mesenchyme were indistinguishable. After 48 hours induction was visible as bright round zones of condensing mesenchyme. After 96 hours zones of condensing mesenchyme had undergone epithelialisation to form complex tubules. At 144 hours epithelial tubular structures and glomeruli indicate a full differentiation of induced tubules in either case.

Various Wnts are sufficient to trigger tubulogenesis

In order to investigate whether *Wnt-4* is sufficient for tubulogenesis, and if this property is shared by other *Wnts* normally expressed in the spinal cord (Parr et al., 1993), we established NIH3T3 cell lines stably expressing various *Wnt* genes and performed direct recombinations between *Wnt* expressing cells and isolated wild-type metanephric mesenchyme. Co-cultures with *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-7a* and *Wnt-7b* expressing cells developed on schedule with those induced by spinal cord, forming complex epithelial tubules with differentiated glomeruli at 144 hours (Fig. 2; Table 2). In contrast, cells expressing *Wnt-5a*, *Wnt-11* or a *lacZ* control did not support survival and differentiation of metanephric mesenchyme (Fig. 2; Table 2). Although we cannot exclude the possibility that the *Wnt-5a* and *Wnt-11* cell lines did not produce the respective Wnt protein, *Wnt* mRNA expression was comparable amongst the various lines.

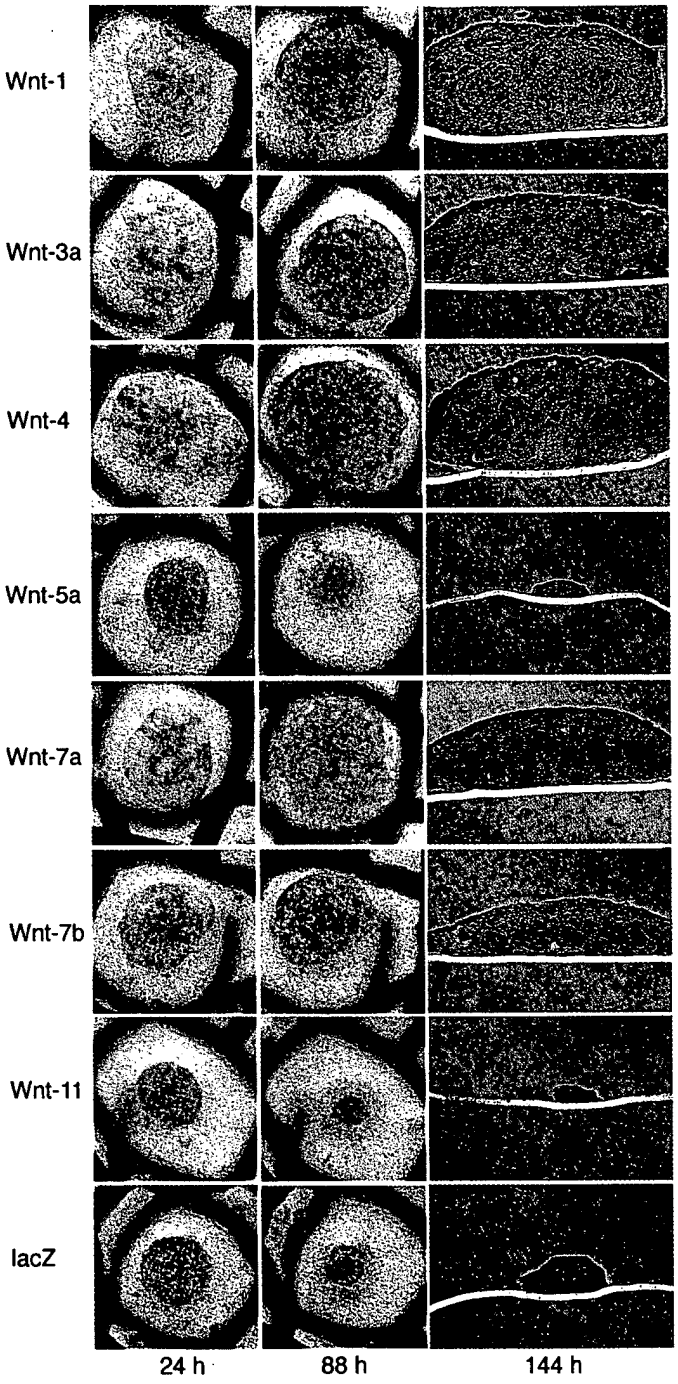

Fig. 2. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing various *Wnt* genes. Brightfield microscopy (24 hours, 88 hours) and histological analysis (144 hours) of direct recombinations between NIH3T3 cells expressing *Wnt* genes and isolated metanephric mesenchyme. After 24 hours bright zones indicating induction are visible in recombinants between wild-type mesenchyme and *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-7a* and *Wnt-7b* expressing cells, respectively. These condensing mesenchymal cells have epithelialised and formed tubular structures after 88 hours. After 144 hours highly elaborate tubular structures are apparent. In contrast, cells expressing *Wnt-5a*, *Wnt-11*, or as a control *lacZ*, respectively, did not support survival and differentiation of metanephric mesenchyme.

Table 2. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing various *Wnt* genes

Cell line	#Induced/#Total
Wnt-1	16/16
Wnt-3a	14/14
Wnt-4	14/14
Wnt-5a	0/12
Wnt-7a	12/12
Wnt-7b	11/12
Wnt-11	0/12
lacZ	1/14
Mesenchyme	1/12

Isolated metanephric mesenchyme from 2-3 11.5 day kidneys was placed on top of NIH3T3 cells expressing various *Wnt* genes. As control mesenchymes were placed on NIH3T3 cells expressing *lacZ* and were placed onto filter without underlying cell layer, respectively. Induction was scored after 6 days using the morphological appearance of the culture (as documented by brightfield microscopy), and histological analysis of selected samples. For each cell type 2-3 independent experiments were performed.

These experiments suggest that a subset of *Wnt* genes, which includes *Wnt-4* and not *Wnt-11*, are able to induce tubule formation. As all of these are expressed in the spinal cord at the time of assay (Parr et al., 1993), it is likely that these signals account for the robust inducing activity of the spinal cord. However, of these *Wnt-4* is the only member which is actually expressed in and which is also required for mesenchymal aggregation (Stark et al., 1994).

Wnt-4 triggers the complete program of tubular differentiation

In order to investigate whether Wnt-4 is sufficient to induce fully developed tubules in isolated metanephric mesenchyme we analysed the induction properties of NIH3T3 cells expressing *Wnt-4* more carefully by assessing the differentiation state of the mesenchyme by histological and molecular criteria. Tubule induction by spinal cord was classically shown to work through polycarbonate filters of a certain pore size (Grobstein, 1956). We seeded *Wnt-4* expressing cells on one filter and separated these cells from isolated mesenchyme by another filter of 1 μm pore size. Induction took place transfilter (Fig. 3), though with a delay when compared with direct recombinants. Further, transfilter cultures where also less compact and flatter. Zones of condensed mesenchyme formed after 24 hours, aggregating mesenchyme and simple epithelial bodies after 48 hours, epithelial tubules after 96 hours and glomeruli by 8 days.

To verify that these morphological features reflected an underlying differentiation of the mesenchyme in response to Wnt-4 we examined the temporal and spatial expression of a number of molecular markers (Fig. 4). *WT-1* was broadly expressed after 1 day refining to small intensely labeled foci by 8 days of culture. This expression profile parallels the expression of this gene during metanephric development (Pritchard-Jones et al., 1990) which is first expressed in condensing mesenchyme, then in simple epithelial bodies before it is restricted to podocytes in the glomeruli. In the recombinants *WT-1* expression seems to mark glomeruli after 8 days in agreement with the histological analysis. Like *WT-1*, *Pax-2* is also broadly expressed after 1 day, but becomes restricted to epithelial bodies and is lost after 4 days reflecting

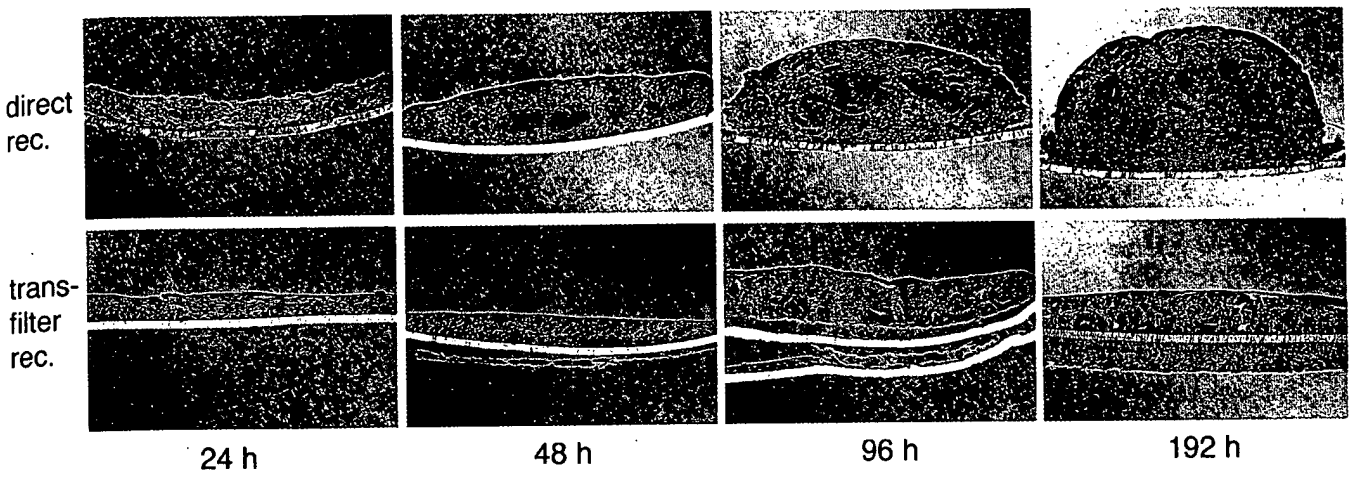

Fig. 3. Histological analysis of tubule induction in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4*. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme directly (direct rec.) and in a transfilter set-up (transfilter rec.), respectively, and analysed by sectioning and histological staining after 24 hours, 48 hours, 96 hours and 192 hours of culture, respectively. Tubule induction in transfilter assays appeared slightly delayed compared to direct recombinations. After 48 hours zones of condensed and aggregated mesenchyme, after 96 hours epithelial tubules were apparent. After 8 days in culture fully differentiated tubular structures including glomeruli were noticed.

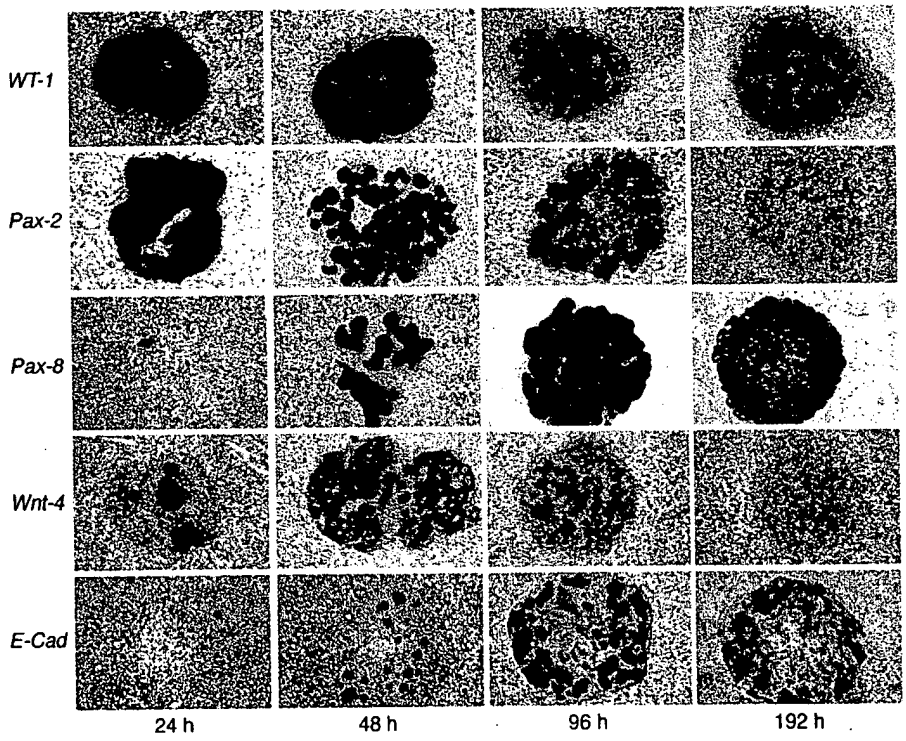

Fig. 4. Marker analysis of tubule induction in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4*. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up and scored for marker expression by in situ analysis after 24 hours, 48 hours, 96 hours and 192 hours of culture, respectively. Expression of *WT-1*, *Pax-2*, *Pax-8*, *Wnt-4* and *E-cadherin*, respectively, were in accordance with expression data known from in vivo and in vitro studies of tubular differentiation. See text for details.

initial expression in condensing metanephric mesenchyme, continuing expression in simple epithelial bodies and subsequent down-regulation as glomeruli start to differentiate (Dressler et al., 1990). *Wnt-4* is expressed in aggregating mesenchyme, in the epithelial bodies which they generate and is subsequently down-regulated as these mature into S-shaped bodies (Stark et al., 1994). *Pax-8*, a paired-box transcription factor, has a similar early expression to *Wnt-4* which has been shown to depend on Wnt-4 activity (Plachov et al., 1990; Stark et al., 1994). In cultures, *Wnt-4* is transiently expressed between 24 hours and 96 hours, peaking at 48 hours. *Pax-8* expression extends longer in S-shaped bodies. *E-cadherin* which is expressed in the proximal tubules in vivo (Vestweber et al., 1985) is present after 24 hours and is maintained consistent with the differentiation of epithelial tubules along the proximal distal axis. Thus, both the molecular and morphological analysis indicate that tubulogenesis in isolated metanephric mesenchyme induced by Wnt-4 follows a similar progression to that observed in the metanephric kidney in vivo. At the stage at which we isolate the metanephric mesenchyme

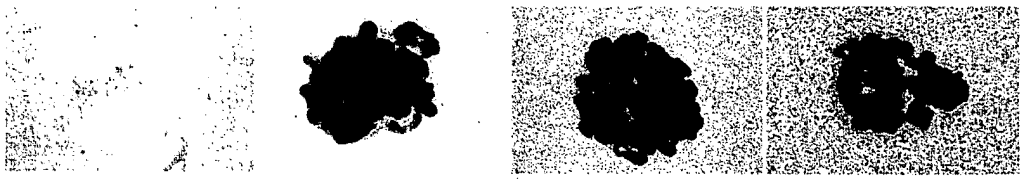

Fig. 5. Pore size dependence of tubule induction by *Wnt-4* expressing cells. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up with various pore sizes of the nucleopore filter. Induction was scored after 4 days by *Pax-8* expression in whole mount in situ analysis. Pore sizes of 0.1 μm and bigger supported full induction of metanephric mesenchyme whereas 0.05 μm pore size dramatically reduced or in most cases abolished induction.

(T-stage of the ureter) initial ureteric signaling has occurred as evidenced by the condensation of mesenchyme around the tip of the ureteric bud. However, this alone is insufficient to support mesenchymal survival and tubulogenesis. In contrast, *Wnt-4* expressing cells are sufficient to support these processes. In order to exclude the possibility that Wnt-4 only maintains *Wnt-4* expression in the isolated mesenchyme we also used mesenchyme derived from 10.75 d p.c. embryos when the ureter bud had just emerged and the metanephric mesenchyme can first be identified. *Wnt-4* expressing cells triggered the complete differentiation program as judged by brightfield observation (12 out of 12 cases) and by molecular criteria (*Pax-8* induction in 8 out of 8 cases after 4 days of culture).

Wnt-4 signaling requires cell contact

We explored tubule induction in isolated metanephric mesenchyme with respect to filter pore size. Experiments using the spinal cord as a heterologous inducer suggest a requirement for cell-cell contact as pore sizes below 0.1 μm, which prevent the extension of cytoplasmic processes, block induction (Saxen, 1987). Transfilter cultures with *Wnt-4* expressing NIH3T3 cells were performed with separating filters ranging from 0.05 μm to 1 μm in pore size, scoring for *Pax-8* induction after 4 days of culture. Pore sizes of 0.1 μm and above supported induction whereas pores of 0.05 μm almost completely abolished induction leading to degeneration of the mesenchyme (Fig. 5; Table 3). Further, we were not able to induce tubulogenesis with supernatants from *Wnt-4* expressing cells (data not shown). Thus, Wnt-4 may act as an insoluble cell bound factor. Such a mode of action agrees well with the known tight association of Wnt proteins with ECM (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Burrus and McMahon, 1995). It also makes it unlikely that Wnt-4 mediated induction occurs through a secondary, soluble factor.

Table 3. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* in transfilter assays with increasing pore size

Pore size (μm)	#Induced/#Total
0.05	3*/13
0.1	14/16
0.4	14/14
0.8	6/6
1	3/3

Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* in a transfilter set-up. Induction was scored after 4 days with in situ hybridisation analysis using *Pax-8* as a probe.

*In each of the specimens scored as induced only 1-4 spots of *Pax-8* expression were seen in contrast to 15-30 with all the other pore sizes.

Wnt-4 signaling requires sulphated glycosaminoglycans

Wnt signaling has been reported to depend on sulfated glycosaminoglycans (GAG)s which might act as cofactors for binding the Wnt protein on the responsive cell (Kispert et al., 1996; Hacker et al., 1997). We were therefore interested to see whether the presence of 30 mM $NaClO_3$, which is known to be a competitive inhibitor of sulfation of GAGs (Kjellen and Lindahl, 1991), may influence induction in our assay. We added this compound at the start of transfilter culture, or 24 and 48 hours later. As a control chlorate was omitted completely. *Pax-8* expression was again scored as a marker for tubule induction after 4 days of culture (Table 4; Fig. 6). When chlorate was added at 0 hours mesenchyme degenerated and *Pax-8* expression was consequently negative. However, addition of chlorate after 24 hours did not influence *Pax-8* expression. Hence, GAGs do not seem to be involved in tubule maturation and differentiation, in agreement with other reports (Davies et al., 1995). Tubule induction does, however, depend on sulfated GAGs in the first 24 hours, the period essential for complete induction by the spinal cord. Although our results point to an important role for GAGs in Wnt-4's action, their precise role remains unclear.

Wnt-4 signaling is only required to trigger tubulogenesis

In order to test whether *Wnt-4* expressing cells can rescue a

Table 4. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* in the presence of 30 mM $NaClO_3$

30 mM $NaClO_3$ added after time in culture (hours)	#Induced/#Total
0	0/19
24	12/19
48	14/17
–	12/15

Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* in a transfilter set-up. 30 mM $NaClO_3$ was added to the medium after the indicated times of setting-up the culture. Induction was scored after 4 days with in situ hybridisation analysis using *Pax-8* as a probe.

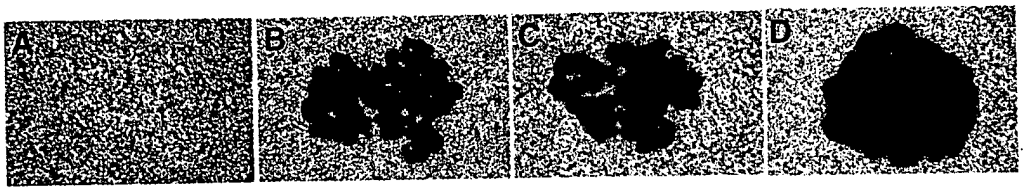

Fig. 6. Glycosaminoglycan dependence of tubule induction by *Wnt-4* expressing cells. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up with addition of 30 mM $NaClO_3$ in the medium. Induction was scored after 4 days by *Pax-8* expression using whole mount in situ hybridisation analysis. Addition of 30 mM $NaClO_3$ after 24 hours (B) or 48 hours (C) of culture did not affect tubule induction compared to untreated controls (D) whereas administration of 30 mM $NaClO_3$ at the beginning of the culture (A) abrogated tubule induction completely.

Wnt-4 mutant mesenchyme we performed direct recombination experiments in culture. Interestingly, *Wnt-4* expressing cells were equally efficient at inducing tubule formation in wild type or *Wnt-4* mutant metanephric mesenchyme (Table 5). Brightfield microscopy and histological analysis of specimen after 6 days in culture revealed the full spectrum of tubular differentiation including glomerulus formation (Fig. 7). Thus, as with spinal cord mediated induction, *Wnt-4* expression in the mesenchyme itself is not required for tubule formation, but supplying *Wnt-4* in adjacent cells is sufficient to trigger the inductive process. These results suggest that whereas *Wnt-4* plays an essential role in initial tubulogenesis, it may not be required for later morphogenesis of the tubule. In agreement with the fact that various *Wnt* genes can trigger tubulogenesis in wild-type mesenchyme *Wnt-1* expressing cells were also sufficient to trigger tubulogenesis in mesenchyme mutant for *Wnt-4* (Table 5).

Table 5. Induction of tubulogenesis in *Wnt-4/Wnt-4* mutant metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* or *Wnt-1*

		#Induced/#Total		
Exp. #	# Recombinants	+/+	*Wnt-4*/+	*Wnt-4*/*Wnt-4*
With NIH3T3 cells expressing *Wnt-4*:				
4	42	7/7	18/18	17/17
With NIH3T3 cells expressing *Wnt-1*:				
2	20	5/5	11/12	3/3

Isolated metanephric mesenchyme from embryos of *Wnt-4*/+ intercrosses was placed on top of NIH3T3 cells expressing *Wnt-4* or *Wnt-1*. Induction was scored after 6 days using the morphological appearance of the culture (as documented by brightfield microscopy).

DISCUSSION

Mammalian metanephric development is a highly coordinated process characterised by a continuous interaction of the epithelial ureter and the surrounding metanephric mesenchyme. Classical organ culture experiments have pointed to the fact that these two compartments achieve coordinated development by use of reciprocal signaling systems. First, the metanephric blastema induces a bud from the adjacent nephric duct which invades and branches into the mesenchyme. This process appears to be mediated by GDNF which is secreted by the metanephric mesenchyme and sensed by the c-ret/GDNFRa receptor complex on the ureter (see Sariola and Sainio, 1997, for review). Next, the metanephric mesenchyme undergoes tubulogenesis upon a permissive stimulus from the ureter.

Since ureter itself is a weak inducer, indeed, ureter has never been proven to induce in transfilter assays of tubule induction, heterologous tissues, most notably dorsal spinal cord, have been used for a long time to mimic ureteric signaling. More recently, several studies have implicated *Wnt* genes in ureteric signaling and in dorsal spinal cord activity. The four key observations are:

(1) *Wnt-11* is expressed in the ureter tips (Kispert et al., 1996).

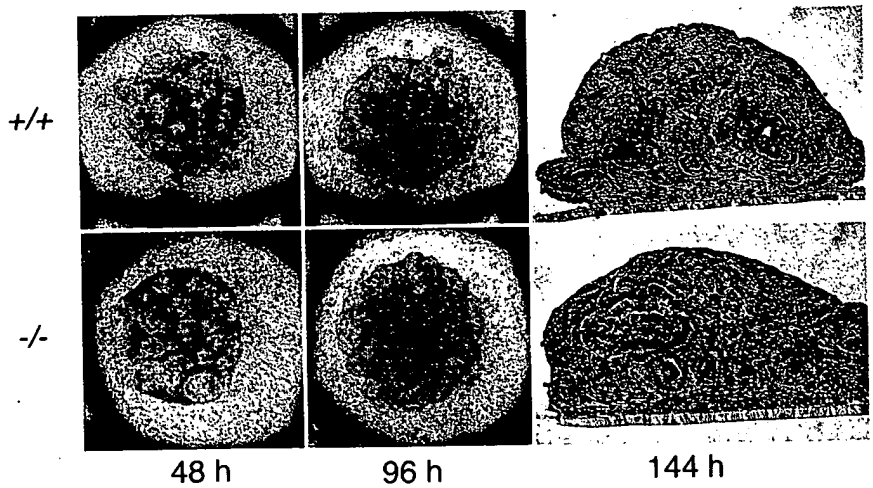

Fig. 7. Induction of tubulogenesis in wild-type and *Wnt-4* mutant metanephric mesenchyme by NIH3T3 cells stably expressing *Wnt-4*. Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* which were supported by a nucleopore filter. After 48 hours and 96 hours cultures were monitored as whole mounts using bright field microscopy, after 144 hours as histological sections. Induction of tubulogenesis in wild-type and *Wnt-4/Wnt-4* mutant metanephric mesenchyme by *Wnt-4* expressing cells were indistinguishable.

(2) Cells expressing *Wnt-1* can induce tubulogenesis (Herzlinger et al., 1994).

(3) *Wnt-4* is a critically required for epithelialisation of condensed mesenchyme (Stark et al., 1994).

(4) Various *Wnt* genes are expressed in the spinal cord (Parr et al., 1993).

With respect to these findings our experimental data are of dual importance. First, they point to the nature of signals emitted by the heterologous inducer spinal cord and clarify the action of these signals with respect to ureter signaling. Second, they argue for a decisive role for Wnt-4 signaling in the mesenchyme, adding another signaling system to tubulogenesis, a process which has not been fully appreciated up to now.

Spinal cord signaling and Wnt-4

Classical kidney organ culture experiments have identified numerous tissues of diverse embryonic origins, which can elicit tubulogenesis in isolated metanephric mesenchyme (Unsworth and Grobstein, 1970; Saxen, 1987). As these tissues are robust inducers, they, rather than the ureter itself, have been widely used to define the process of kidney tubule induction. In our recombination experiments we have demonstrated that these inducers, exemplified by dorsal spinal cord tissue, can trigger tubulogenesis in *Wnt-4* mutant mesenchyme. Therefore, it seems unlikely that the signals emitted from the spinal cord, and possibly other heterologous inducing tissues, mimic an endogenous ureteric signal(s) which would still require *Wnt-4* for tubule formation. We suggest they act on the level of *Wnt-4* in the mesenchyme. Since dorsal spinal cord used in the assays was also mutant for *Wnt-4* other *Wnts* may replace Wnt-4 activity in the mesenchyme. By using cell lines expressing various *Wnt* genes we show that *Wnt-1*, *Wnt-3a*, *Wnt-7a*, *Wnt-7b*, all of which are expressed in spinal cord, can evoke tubulogenesis in isolated metanephric mesenchyme. The additive action of several Wnt proteins may also explain why dorsal spinal cord represents such a strong source of inducer. Further, *Wnts* are widely expressed during embryogenesis (Parr et al., 1993; Lee et al., 1995) and could explain why so many tissues can trigger tubulogenesis in isolated metanephric mesenchyme.

In summary, our results suggest a different interpretation of the use of kidney cultures to elucidate the nature of the ureteric signal involved in inducing the mesenchyme. Experiments which have used heterologous sources of tubule inducers, most notably the spinal cord, may not have been investigating the nature of ureteric signaling, but rather the mesenchymal action of signals such as Wnt-4. At present, the exact nature of ureteric signaling remains obscure. It seems clear that a primary signal from the ureter leads to survival and initial condensation of metanephric mesenchyme. This primary signal might be required for a sufficient length of time to allow auto-induction of the mesenchyme by Wnt-4. Alternatively, a secondary signal from the ureter tip might be necessary to induce *Wnt-4* expression in aggregating mesenchyme.

Our experiments argue against a role for Wnt-11 as a ureteric signal for mesenchymal aggregation. As a secreted glycoprotein expressed at the ureter tip Wnt-11 was a prime candidate for such an activity. However, we were not able to get tubulogenesis with cells expressing *Wnt-11*. Recent loss-of-function experiments also support this notion. Mice homozygous for a likely null mutation of *Wnt-11* do not exhibit an overt kidney phenotype (S. Vainio, A. Kispert and A. P. McMahon, unpublished observation). At this time, the function of *Wnt-11* is unclear.

Wnt-4 is a mesenchymal signal for tubulogenesis

Analysis of *Wnt-4* mutants has demonstrated a critical role for *Wnt-4* in kidney development. Homozygous pups die 24 hours after birth due to small agenic kidneys consisting of undifferentiated mesenchyme intermingled with collecting duct tissue. Histological and marker analysis revealed that primary condensation of mesenchymal cells around the ureter tips as well as ureteric branching occurs normally. However, mutant kidneys quickly become growth retarded and the mesenchyme remains undifferentiated lacking pretubular cell aggregates and epithelial tubules. Since kidney size as well as cell death initially remain unaffected, proliferation is unlikely to be controlled by *Wnt-4*. Rather, the lack of *Wnt-4* expression itself and of epithelial structures in the mutant mesenchyme argues that Wnt-4 may autoinduce the epithelialisation of condensed mesenchyme. In this study we have shown that mesenchymally derived *Wnt-4* is not only required but also sufficient for induction of tubulogenesis in the mammalian kidney. Judging by histological and molecular markers Wnt-4 can elicit the complete program of tubular differentiation in isolated metanephric mesenchyme. The activity of Wnt-4 contrasts with other factors thought to regulate mesenchymal development. For example, FGF (Perantoni et al., 1995) and EGF (Weller et al., 1991; Koseki et al., 1992) can both support mesenchymal survival but are not sufficient for tubulogenesis. Like Wnt-4, BMP-7 has been suggested to induce tubules (Vukicevic et al., 1996) but loss-of-function studies indicate it is not essential for tubule formation in vivo as some glomeruli form in *BMP7* mutants (Dudley et al., 1995; Luo et al., 1995). In contrast, loss of *Wnt-4* leads to a complete absence of glomeruli.

Wnt-4 activity shows all the characteristics which have previously been ascribed to induction by dorsal spinal cord tissue. Signaling is cell-contact dependent. Below a certain pore size in the transfilter assay the formation of cellular processes which penetrate the filter pores is inhibited and isolated mesenchyme degenerates. Cell contact dependence agrees well with the fact that Wnt proteins interact with extracellular matrix (ECM) components (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Burrus, 1994; Burrus and McMahon, 1995). The chlorate inhibition experiments defines a critical period of 24 hours for induction. This is in agreement with classical studies which showed that the inducer tissue can be removed after this time with tubulogenesis proceeding undisturbed (Saxen, 1987). Possibly, every cell has to get in contact with the inducer to initiate tubulogenesis. We suggest that further differentiation, i.e. aggregation and epithelialisation of mesenchymal cells is only initiated when a certain number of cells (a small community) has received the Wnt-4 signal. At this time mesenchymal development is independent of ureteric signaling.

Chlorate acts as a competitive inhibitor of sulphotransferases and inhibits the sulphation of glycosaminoglycans (Kjellen and Lindahl, 1991). Our inhibition studies point to a critical role of these ECM compounds in tubulogenesis. Numerous studies have shown that branching morphogenesis of the ureter as well

as branching of other epithelia requires an intact ECM (Platt et al., 1987; Davies et al., 1995; Roskelly et al., 1995; Kispert et al., 1996). Since presence of chlorate after 24 hours does not influence tubulogenesis GAGs do not seem to be involved in tubule maturation and differentiation, in agreement with other reports (Davies et al., 1995). Tubule induction does, however, depend on sulfated GAGs in the first 24 hours, the period essential for complete induction by the spinal cord. Although our results point to an important role for GAGs in Wnt-4's action, their precise role remains unclear. It is possible that chlorate acts on the Wnt secreting cell. Recent experiments in the fly which indicate that GAG synthesis is also required for *wingless* (*Drosophila Wnt-1*) signaling (Reichsman et al., 1996; Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) suggest that GAGs are required in the responsive cell for signal transduction. Most likely as is the case for FGFs, GAGs may act as co-receptors, facilitating presentation or increasing the local concentration of the ligand.

Wnt-4 expression in the metanephric mesenchyme is initiated in the aggregating mesenchyme and maintained in the comma shaped bodies before it is downregulated in S-shaped bodies. Therefore, *Wnt-4* might have a later function in tubulogenesis which is masked in the earlier requirement to form a tubule. However, cells expressing *Wnt-4* are sufficient to induce tubulogenesis in *Wnt-4* mutant mesenchyme arguing that *Wnt-4* expression may not have a later role. It seems that Wnt-4 probably acts as a trigger to start an intrinsic program in the mesenchymal cells which then proceed to form complex nephron like structures. From our experiments it is not clear whether in vivo Wnt-4 acts in an autocrine or a paracrine fashion. However, it seems clear that the signal acts very locally. Interestingly, although these experiments demonstrate that *Wnt-4* is itself a target of its own signaling activity, induced metanephric mesenchyme is not able to induce tubule formation when recombined with uninduced mesenchyme. This lack of homeogenetic induction could be taken as evidence against Wnt-4 acting as a mesenchymally derived tubule inducer. However, there are many other explanations for this result. For example lateral inhibition mechanism as is commonly used in fine grained patterning may limit Wnt-4 action. Alternatively, levels of induced Wnt-4 may not be high enough to induce more mesenchymal cells. More directly, a number of potential Wnt antagonists have been described that could potentially modulate Wnt-4 signaling (Moon et al., 1997; Rattner et al., 1997; Glinka et al., 1998).

Our view is that kidney tubulogenesis is a multi-step process with a hierarchy of signaling systems. A permissive signal from the ureter to the mesenchyme triggers survival and tubulogenesis in the mesenchyme, signals from the mesenchyme to the ureter are required for proliferation and branching morphogenesis of the ureter. Most likely, other signaling systems within the ureter are required for local adhesion and proliferation changes which may mediate branching morphogenesis, and within the mesenchyme for tubulogenesis as evidenced by the role of *Wnt-4*. Taking the complexity of developmental events in the mesenchyme into account, it is conceivable that additional signaling systems control the ratio between interstitial and metanephrogenic cells, between condensing and non-condensing cells, and the maintenance of the mesenchymal stem cells in the periphery. Undoubtedly, the functional analysis of other secreted factors will shed light on these diverse events in kidney development.

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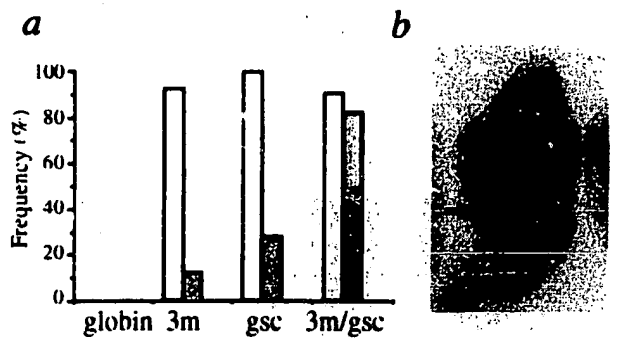

FIG. 4 Secondary axis formation. *a*, Incidence of ectopic somite and notochord formation in embryos injected with *Xlim-1* mutant *3m*, *gsc*, or both. Open bars, ectopic somite; grey bars, one ectopic notochord; black bar, two ectopic notochords. The *3m*-induced secondary axes also contained a high incidence of neural tissue and cement gland (not shown). The total number examined was 28 (globin), 100 (3m), 72 (gsc) and 39 (3m/gsc). *b*, Example of two ectopic notochords formed in an embryo coinjected with *3m* and *gsc* RNAs, as visualized by whole-mount staining with the notochord-specific antibody Tor70[27] at stage 27/28. Dosal view, anterior is up; arrows indicate ectopic notochords.
METHODS. Embryos were injected with RNA into two blastomeres at the 4-cell stage in the ventral equatorial region. The amount of RNA was 0.5–1.75 (globin), 0.5–1.0 (*3m*) and 0.38–0.75 (*gsc*) ng per embryo. In coinjection of *3m* and *gsc*, the ratio of *3m* to *gsc* was 1 to 0.75. At tailbud stages, embryos were analysed by whole-amount immunostaining[28] after bleaching with 10% H_2O_2. In some injected embryos, blastopore closure was incomplete, probably due to gastrulation arrest; such embryos were discarded.

are displayed by *gsc* when its mRNA is injected into the ventral equatorial region[3,14], but not in animal ectodermal cells (ref. 15 and our results). Cells expressing *3m* send inducing signals to adjacent cells (Fig. 2); therefore, it is likely that LIM domain mutants of *Xlim-1* activate genes encoding neural- and muscle-inducing factors. These putative molecules appear to be distinct from noggin or follistatin (Fig. 3*a*), implicating new signalling factors in organizer function. □

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Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by *Wnt-4*

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THE kidney has been widely exploited as a model system for the study of tissue inductions regulating vertebrate organogenesis[1,2]. Kidney development is initiated by the ingrowth of the Wolffian duct-derived ureteric bud into the presumptive kidney mesenchyme. In response to a signal from the ureter, mesenchymal cells condense, aggregate into pretubular clusters and undergo an epithelial conversion generating a simple tubule. This then undergoes morphogenesis and is transformed into the excretory system of the kidney, the nephron. We report here that the expression of *Wnt-4*, which encodes a secreted glycoprotein, correlates with, and is required for, kidney tubulogenesis. Mice lacking *Wnt-4* activity fail to form pretubular cell aggregates; however, other aspects of mesenchymal and ureteric development are unaffected. Thus, *Wnt-4* appears to act as an autoinducer of the mesenchyme to epithelial transition that underlies nephron development.

Wnt-signalling is thought to play diverse roles, regulating pattern, cell fate choices and mitotic activity in different embryonic tissues. To address the possibility that Wnt-family members may participate in development of the vertebrate kidney, we examined expression of 12 mouse Wnt genes by *in situ* hybridization (data not shown). One of these, *Wnt-4*, was expressed in the kidney mesenchyme and its derivatives. *Wnt-4* messenger RNA was detected in condensed mesenchymal cells on both sides of the stalk of the ureter at 11.5 days postcoitum (d.p.c.) and correlated to the site where the first pretubular cell aggregates form[2] (Fig. 1*a–c*). During subsequent development, this expression pattern was repeated in newly forming aggregates, as well as their simple epithelial tubular derivatives (Fig. 1*d–f*).

Wnt-4 expression was also detected in comma-shaped bodies, but later became restricted in descendent S-shaped bodies to the region where epithelial fusion was occurring with the collecting duct, and was lost after fusion was completed (Fig. 1*e*, *f* and data not shown). At later stages, *Wnt-4* expression became confined to the periphery of the kidney where new tubules were forming (Fig. 1*f*). Interestingly, in both the chick (Fig. 1*g*) and mouse[3] (data not shown), the dorsal spinal cord, a potent inducer of kidney tubule differentiation[1,2], also expressed *Wnt-4*.

Development of the metanephric kidney is preceded by the formation of a mesonephric kidney. In the mouse, this is a vestigial structure, whereas in avian species, it is an elaborate functional organ during embryonic life. *Wnt-4* was expressed throughout mesonephric tubulogenesis from the aggregating mesenchyme until fusion of the epithelial tubules with the Wolffian (mesonephric) duct (Fig. 1*g*, *h*). Thus, it is likely that the role of *Wnt-4* is conserved in the development of the mesonephric and metanephric kidneys.

To examine *Wnt-4* function, we used gene targeting in mouse embryonic stem (ES) cells to replace the third coding exon of the *Wnt-4* gene with a selection cassette containing neomycin phosphotransferase (neo) (Fig. 2), thereby generating a likely

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null allele. Adult mice heterozygous for this disrupted allele showed no obvious phenotype. However, homozygous pups died within 24 h of birth. As *Wnt-4*$^{-/-}$ embryos were recovered at the expected mendelian frequency at late stages of gestation (data not shown), *Wnt-4* is not essential for *in utero* development. All neonatal and 18.5 d.p.c. *Wnt-4*$^{-/-}$ embryos contained small agenic kidneys (compare with adjoining adrenal gland in Fig. 3*a*, *b*), consisting of undifferentiated mesenchyme interspersed with branches of collecting duct epithelium (Fig. 3*c*–*f*). Thus, death of homozygous pups is almost certainly due to the lack of kidney function.

To address the role of *Wnt-4*, we analysed kidney development in mutant embryos. As expected, initial development of the primary condensate around the tips of the ureteric bud appeared histologically normal (Fig. 3*g*, *h*), suggesting that induction of kidney mesenchyme by the ureter was unaffected. Early on the 15th day of pregnancy, no gross difference was observed in the size of kidneys between *Wnt-4*$^{+/-}$ and *Wnt-4*$^{-/-}$ embryos (Fig. 3*i*–*l*). Like their normal litter mates, mutant embryos had undergone considerable branching of the ureteric epithelium. However, the kidney mesenchyme remained morphologically undifferentiated, lacking pretubular aggregates and more developed tubules (Fig. 3*j*, *l*). Later on the same day, *Wnt-4*$^{-/-}$ kidneys were clearly growth retarded (Fig. 3*m*, *n*) and the mesenchyme persisted in a morphologically undifferentiated state (data not shown). Three out of ten *Wnt-4*$^{-/-}$ embryos examined on the 15th day of pregnancy showed no histological evidence of mesenchymal aggregation. In the remainder, a few poorly developed aggregates were observed (less than 10% of the number of aggregates scored in *Wnt-4*$^{+/+}$ or *Wnt-4*$^{+/-}$ litter mates). We never observed comma-shaped or S-shaped bodies.

Kidney development was further investigated by the analysis of a variety of molecular markers. Presumptive and induced kidney mesenchyme, as well as differentiating glomerulae, express the *Wilms tumor* (*WT-1*) before mesenchymal condensation[4,5]. Initial expression of *WT-1* was unchanged in *Wnt-4*$^{-/-}$ embryos (Fig. 4*a*, *b*), persisting until at least 14.5 d.p.c. However, throughout this period, expression was only observed in the mesenchyme (Fig. 4*c*, *d*). *N-Myc*, an early marker of tubule induction, is first detected in a subpopulation of condensed mesenchyme and is then upregulated in epithelial structures which presumably arise from these cells[6]. *Pax2* has also been suggested to regulate induction and epithelial conversion of the kidney mesenchyme[7,8]. *N-Myc* and *Pax2* were expressed until at least 14.5 d.p.c. in *Wnt-4*$^{-/-}$ embryos; however, no expression was detected in tubules (Fig. 4*e*–*l*). These data suggest that the mesenchymal cells fated to form tubules were induced but failed to convert into epithelial structures.

Failure of pretubular aggregation is also supported by analysis of *Wnt-4* and *Pax-8* expression. Although *Wnt-4* expression appears to precede that of *Pax8* (data not shown) both genes share an identical distribution in the pretubular aggregate and

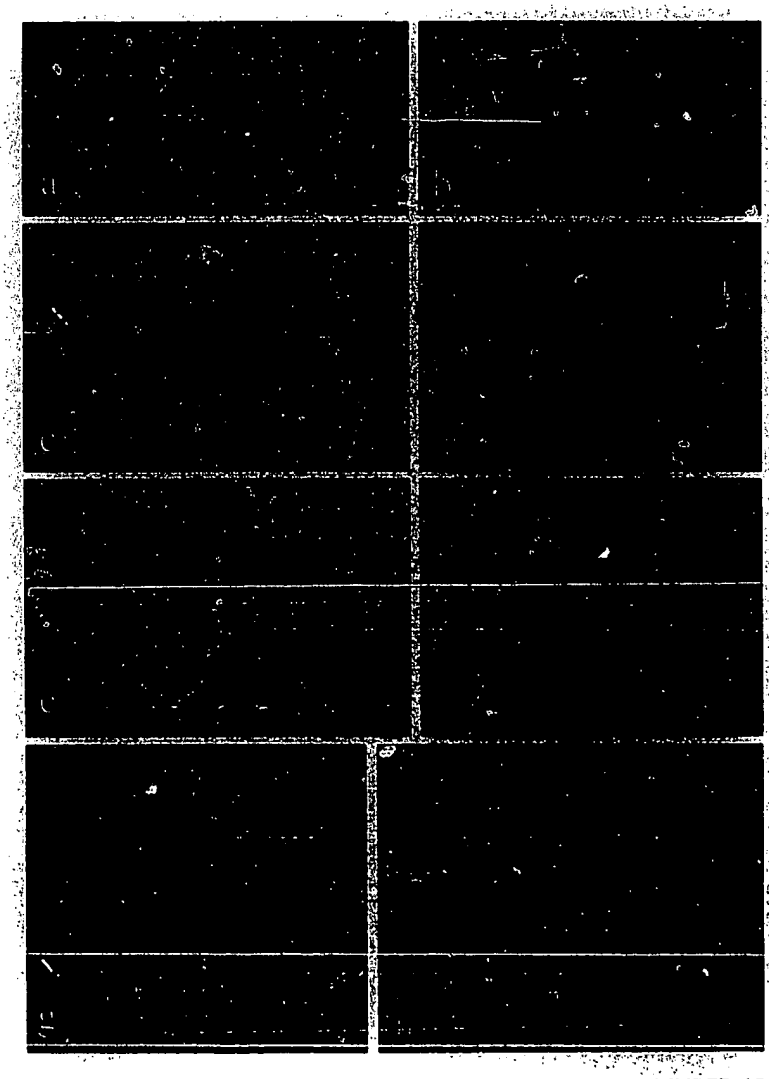


FIG. 1 Expression of *Wnt-4* during morphogenesis of the metanephric kidney of the mouse and mesonephric kidney of the chick. *In situ* hybridization with ^{35}S mRNA probes was used to detect mouse (*a*–*f*) and chick (*g*, *h*) *Wnt-4* mRNA. *a*, In the metanephric kidney *Wnt-4* expression is first seen at 11.5 d.p.c., in condensed mesenchymal (cm) cells on both sides of the invading ureteric bud (ub), preceding the formation of the first pretubular aggregates. *b*, A transverse section through a similar stage kidney to that shown in *a*. *c*, *Wnt-4* expression becomes restricted to pretubular aggregates on initiation of ureteric bud branching (12.5 d.p.c.). Expression is also seen in the mesonephric mesenchyme (arrowheads), adjacent to the mesonephric duct (arrow). *d*, By 13.5 d.p.c., *Wnt-4* expression is apparent in primitive tubular aggregates. *e*, On subsequent branching of the ureter (14.5 d.p.c.), *Wnt-4* expression is induced in the assembling aggregates. Expression is also seen in comma-shaped bodies (arrow), and more advanced stages of tubular morphogenesis. *f*, Later during development (16.5 d.p.c.) *Wnt-4* expression is confined to the cortex of the kidney where new tubular aggregates are still forming. *g*, In the stage 20 chick embryo, *Wnt-4* expression is induced in the mesonephric mesenchyme of the mesonephros, adjacent to the mesonephric duct (arrow). *Wnt-4* is also expressed in a graded distribution in the dorsal half of the spinal cord (sc). *h*, At stage 27–29, development of the mesonephric kidney is advanced and *Wnt-4* expression is apparent in early stage aggregates (arrowheads), as well as in more advanced stages. By the time that the tubules fuse with the mesonephric duct, *Wnt-4* expression becomes restricted to the tip of the tubule at the point of fusion (arrow) and is lost after fusion. Scale bars, 10 μm (*a*–*c*, *g*, *h*); 100 μm (*f*).

METHODS. *In situ* hybridization analysis was according to published procedures[24] using a *Wnt-4* mRNA probe[3]. The chick *Wnt-4* cDNA consisted of a roughly 400 base pair fragment generated by polymerase chain reaction (J. McMahon and A.P.M., unpublished data).

developing tubule[9] (compare Fig. 4*o* and *s*). In mutant embryos, expression of both of these genes was never detected in kidneys scored as completely negative for tubule formation by histological analysis and was only detected in the rare case of a small and poorly organized aggregate (Fig. 4*m–t*). As normal *Wnt-4* expression was observed in several tissues that were not overtly affected in *Wnt-4*$^{-/-}$ embryos (floorplate, spinal cord and mesonephric mesenchyme; Fig. 4*n* and data not shown), it is clear that the targeted allele was not transcribed. Thus, the failure to detect cells initiating *Wnt-4* expression suggests that Wnt-4 may be required for full activation of its own expression in the kidney mesenchyme. Alternatively, cells lacking Wnt-4 activity might rapidly adopt another fate. Further, *Wnt-4* is required, directly or indirectly, for the activation and/or maintenance of *Pax8* expression. Activation of *Pax-8*, a paired domain containing transcription factor, may be critical for the regulation of other genes essential for tubule formation.

Kidney development is critically dependent on the ureter. For example, mice lacking *c-ret* activity, a receptor protein tyrosine kinase normally expressed in the ureteric bud[10], die because of complete or partial failure of kidney development[11]. Expression of c-*ret* and c-*ros*, a second receptor tyrosine kinase transcribed in the ureteric epithelium[12,13], was activated and maintained in the tips of the ureteric bud in mutant kidneys (Fig. 4*u–y*; data not shown). This result, together with the observed branching of the ureter, supports the conclusion that development of the

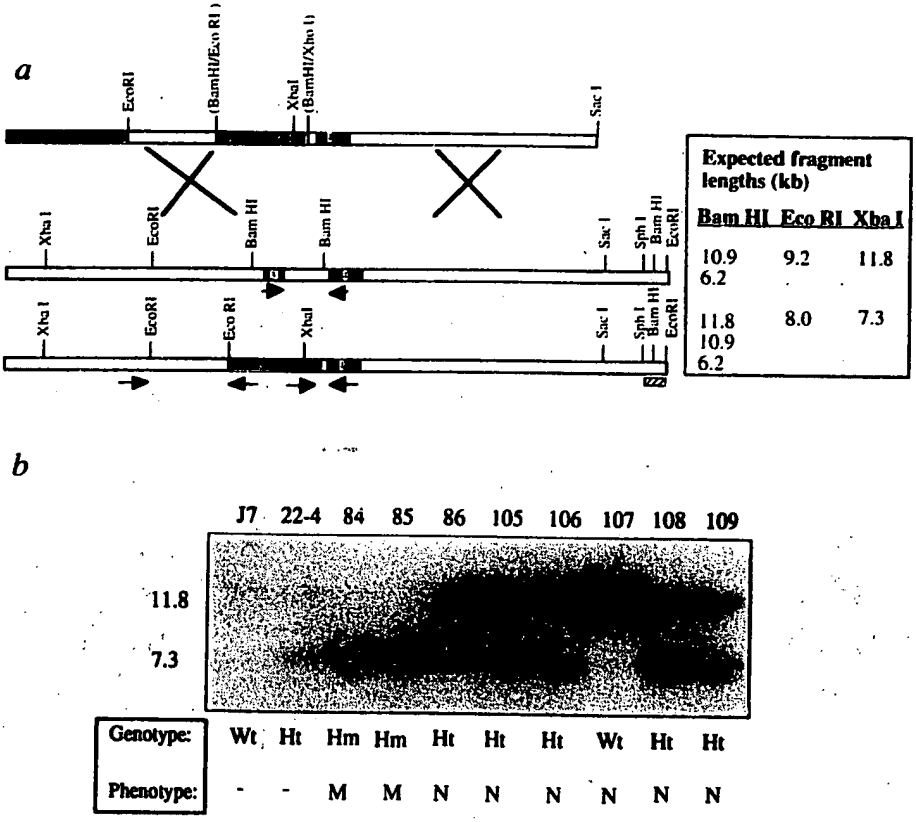


FIG. 2 Gene targeting at the *Wnt-4* locus. *a*, Schematic representation of the *Wnt-4* targeting vector (upper), *Wnt-4* locus (middle) and expected recombination event (lower). Successful gene replacement by the targeting vector results in the deletion of roughly 2.2 kb of *Wnt-4* genomic sequence, including all of the coding sequence derived from exon 3 (amino acids 106 to 196 in the predicted Wnt-4 protein sequence[26]), and insertion of a *PGK-neo* selection cassette[28], in the same transcriptional orientation as that of the *Wnt-4* gene. The arrows indicate PCR primers, and the hatched box a DNA probe used to identify putative targeted ES cell clones. *b*, Southern blot analysis of gene targeting at the *Wnt-4* locus and transmission of the targeted allele. An 11.8 kb *Xba*I fragment, indicative of the wild type *Wnt-4* allele, is detected in the parental CJ7 ES cell line[27]. An additional fragment of 7.3 kb, corresponding to the targeted allele, is detected in DNA from clone 22-4. Additional Southern analysis with *Bam*HI and *Eco*RI digests, and with a 5′ flanking probe, confirmed that the predicted targeting event occurred in clone 22-4 (data not shown). Lanes 84 to 109 represent selected DNA samples from 18.5 d.p.c. progeny derived from intercrosses between mice heterozygous for the targeted *Wnt-4* allele. Agenesis of the kidney was apparent in all homozygous (Hm) embryos at this stage. No phenotype was observed in heterozygous (Ht) or wild-type (Wt) littermates.

METHODS. The gene targeting construct, containing 1.6 kb of 5′ and 4.5 kb of 3′ homology, was transfected into the CJ7 ES cell line[27] and clones selected using positive (*PGK-neo* cassette[26]) and negative (*MC1TK* cassette[28]) selection with G418 and FIAU, respectively, as described previously[29]. Surviving clones were screened for homologous recombination by PCR using a 5′ oligonucleotide homologous to sequences in intron 2 of *Wnt-4* (5′-GCGTTCTGCCTCCCTCCTGCGGGG-3′), which lies just upstream of the 5′ homology region in the targeting construct, and a 3′ oligonucleotide (5′-GGGAGCCGGTTGGCGCTACCGGTGG-3′) homologous to PGK promotor sequences in the *PGK-neo* cassette (arrows in *a*). Sample preparation and PCR amplification was as described previously[29], except that the initial PCR was performed on pools of 5 samples. Gene replacement at the *Wnt-4* locus leads to the amplification of a 1.6 kb 5′ homology region. Because a band of the expected size was not observed with ethidium bromide staining, the PCR products were transferred to nylon membranes and hybridized with a ^{32}P-labelled oligonucleotide homologous to sequences within the amplified region (5′-GCTGAGTGGCTAGAGCCCAG-3′). One positive pool (22) was identified; on further analysis, one sample of the five clones in this pool was PCR positive. The overall targeting frequency was ~1 in 200 clones which survived double selection. DNA was prepared from this clone (22-4) and analysed for correct targeting by Southern blot analysis using both 5′ and 3′ single copy probes and multiple diagnostic restriction enzyme digests. Having verified that homologous recombination had occurred as expected, 22-4 cells were used to generate chimaeras and germ-line transmission of the targeted allele was monitored by Southern analysis using the indicated probe (hatched box in *a*). All subsequent genotyping was done by Southern blot or by PCR. The wild-type *Wnt-4* allele was detected by PCR using a 5′ oligonucleotide (5′-CTTCACAACAACGAGGCTGGCAGG-3′) and a 3′ oligonucleotide (5′-CACCCGCATGTGTGTCAAGATGG-3′) homologous to nucleotides 655–679 in exon 3 and 683–706 in exon 4 of the *Wnt-4* cDNA[25]. The targeted *Wnt-4* allele was identified by using a 3′ primer homologous to sequences within the *PGK-neo* cassette (5′-GCATTGTCTGAGTAGGTGTCATTC-3′) and the *Wnt-4* exon 4 primer described above. PCR amplifies fragments of about 400 bp (targeted allele) and 700 bp (wild-type allele). PCR was done for 45 cycles of: 1 min at 93 °C, 65 °C for 30 s, 72 °C for 30 s, followed by 5 min at 72 °C.

ureteric epithelium, at these stages, is independent of both *Wnt-4* expression and tubule formation.

Up to the 15th day of pregnancy, there is no visible decrease in size or increase in cell death in mutant kidneys. This period is normally marked by a general decrease in cell proliferation in developing tubules[2]. Thus, it seems unlikely that Wnt-4 regulates cell growth or survival. Together, our evidence is most consistent with a model in which *Wnt-4* acts as an autoactivator of the mesenchymal to epithelial transition. Thus, initiation of *Wnt-4* expression in a cluster of induced cells leads to these same cells forming the pretubular aggregate. Continued expression of *Wnt-4* suggests an additional role in later stages of tubule morphogenesis which cannot be addressed at present.

Clearly, some mechanism must account for the discrete localization of *Wnt-4* to the aggregates. One possibility is that induction of mesenchyme by the ureter leads to both the activation of *Wnt-4* expression and the competence to respond to this signal. Thus, only those cells in the distal mesenchyme that were previously in contact with the ureter are induced to aggregate by a Wnt-4 signal. Indeed, aggregating cells are unable to elicit tubule formation in uninduced nephrogenic mesenchyme[14]. Alternatively, aggregating mesenchymal cells may inhibit neighbouring cells from adopting the same fates.

One likely step in the mesenchyme to epithelial transition is regulation of cell adhesion. Interestingly, ectopic expression of *Wnt-4* (R. Moon, personal communication) and *Wnt-5a*, in the *Xenopus* embryo[15], may increase cell adhesiveness. Moreover, *armadillo*, which is a *Drosophila* homologue of beta-catenin, a cytoplasmic protein essential for E-cadherin-mediated adhesion[16,17], is required for *wingless* (the *Drosophila* orthologue of *Wnt-1*) signalling[18,19]. *Wnt-1* has been shown to modulate cell adhesion *in vitro*[20,21], and the expression of the cell adhesion molecule, E-cadherin *in vivo*[22]. Interestingly, E-cadherin is expressed in the early kidney tubule, after activation of *Wnt-4* and *Pax-8* expression[23]. Hence, it is tempting to speculate that

FIG. 4 Expression of mesenchymal and epithelial marker genes in kidneys of *Wnt-4* mutants. *WT-1* is expressed in the undifferentiated nephrogenic mesenchyme (nm) at 12.5 (*a*) and 14.5 (*c*) d.p.c. in *Wnt-4*$^{+/-}$ or $^{+/-}$ embryos. Expression is also seen in early and late stages of tubule morphogenesis at 14.5 d.p.c. (arrowheads in *c*). *WT-1* expression in the kidneys of *Wnt-4*$^{-/-}$ embryos is restricted to morphologically undifferentiated mesenchyme (*b*, *d*). *N-Myc* expression at 12.5 d.p.c. localizes to a subset of induced mesenchyme in kidneys from wild-type (*c*) and homozygous mutant embryos (*f*). Expression is also seen in more advanced stages of tubule development in the normal kidney (*g*), but not in kidneys from *Wnt-4*$^{-/-}$ embryos (*h*). *Pax2* shows complex expression, first in the invading ureteric epithelium and condensed mesenchyme (*i*), then also in comma- and S-shaped bodies (arrowheads), and proximal and distal collecting duct (cd) epithelium, at 14.5 d.p.c. (*k*). Expression is unchanged in the ureter and kidney mesenchyme of *Wnt-4* mutants at 12.5 d.p.c. (*j*), and extends into the collecting duct epithelium at 14.5 d.p.c. (*l*); however, epithelial expression is limited to ureteric derivatives. *Wnt-4* (*m–p*) and *Pax-8* (*q–t*) are both expressed in pre- and early tubular aggregates (*m*, *q*), as well as in comma- and S-shaped bodies (*o*, *s*). In *Wnt-4* mutants only occasional expression of either gene is seen in the kidney and expression is restricted to poorly developed aggregates (arrowed in *p* and *t*). *o* and *s* are adjacent sections. *Wnt-4* expression in the central nervous system and mesonephric mesenchyme (ms in *n*) is unaltered in *Wnt-4*$^{-/-}$ embryos. Expression of *c-ret* localizes to the tips of the ureter at 12.5 (*u*) and 14.5 (*x*) d.p.c. In *Wnt-4* mutants, expression persists at the tips of the newly branched ureter at 12.5 (*v*) and until at least 14.5 d.p.c. (*y*), suggesting that development of the ureteric bud is not dependent on Wnt-4. Scale bar, 10 μm.

METHODS. *In situ* hybridization to embryo sections was as described[24]. The probes used were as described: *WT-1*[5], *N-myc*[30], *Pax-2*[7], *Wnt-4*[3], *Pax-8*[9], *c-Ret*[10].

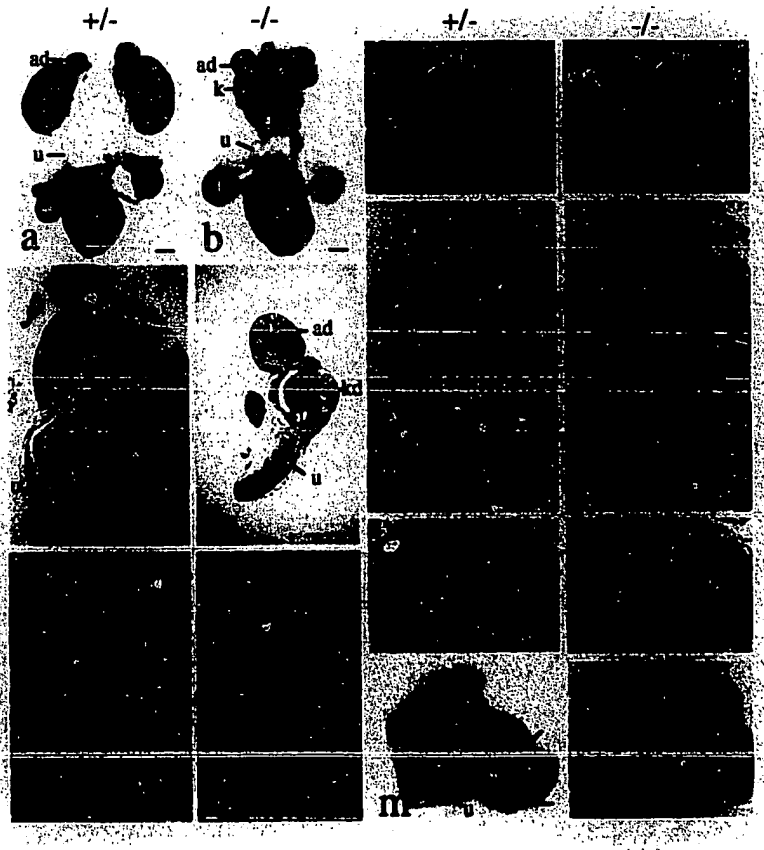
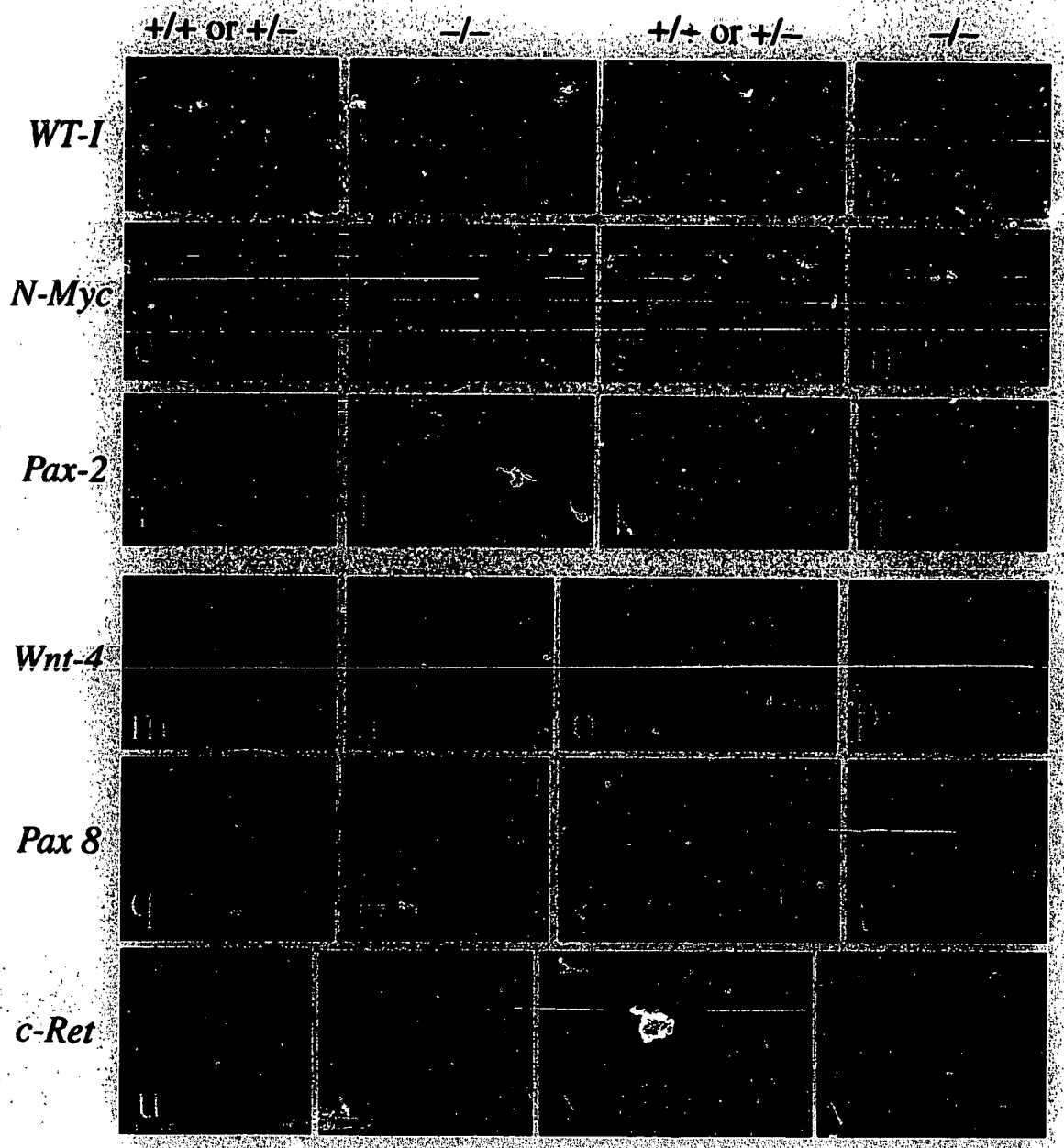


FIG. 3 Histological analysis of kidney development in *Wnt-4* mutant embryos. Whole-mount views (*a*, *b*, *m*, *n*) and histological sections (*c–l*) of kidneys from embryos taken at 18.5 d.p.c. (*a–f*), 12.5 d.p.c. (*g*, *h*), and early (*i–l*) and late (*m*, *n*) on the 15th day of pregnancy. *e*, *f*, High-power views of *c* and *d*, respectively. *k*, *l*, High-power views of *i* and *j*, respectively. For description, see text. Arrows indicate the invading and branching ureteric epithelium; arrowheads, epithelial tubular aggregates. ad, Adrenal gland; bl, bladder; cm, condensed mesenchyme; gl, glomerulus; kd, kidney; ov, ovary; t, testis; u, ureter. Scale bars, *e*, *f*, *g*, *h*, *k*, *l*, 10 μm; *a–d*, *i*, *j*, *m*, *n*, 100 μm.

METHODS. Embryos were collected into PBS, a sample of the yolk sac or liver was removed for genotyping and the remaining embryo, or isolated kidneys, were fixed in Bouin's fixative. Samples were dehydrated, embedded in wax and sectioned at 3 to 6 μm. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.



Wnt-4 may regulate mesenchymal aggregation and tubule formation through modulation of cell adhesion factors. □

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A Wnt-Wnt Situation

Meeting Review

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A recent Juan March Foundation workshop on "*wnt* genes and Wnt signaling" brought developmental and cancer biologists together to share some of the latest advances in Wnt research. Discussion topics included molecular, genetic, and genomic dissections of *wnt* genes in embryogenesis and cancer, Wnt signaling components and downstream targets, interactions with other signaling pathways, cell biological aspects of Wnt signaling, and a first glimpse of a purified Wnt protein.

Since the discovery 21 years ago of the first *wnt* gene, *wnt-1* (*int-1*), as an oncogene that causes mouse mammary tumors (Nusse and Varmus, 1982), investigation on *wnt* genes and Wnt signaling pathways has blossomed into a lively field on its own and infiltrated many corners of developmental biology and cancer research. *wnt* genes encode secreted cysteine-rich signaling proteins that regulate many cell behaviors, including proliferation, differentiation, survival, polarity, and movement. *wnt* genes have been found throughout the animal kingdom and perform an astonishing array of functions, from the establishment of the basic body plan and the generation of various organs and tissues to axon guidance and synapse formation. Connections between Wnt signaling and human disease have been solidified by studies of malignancies, such as colorectal cancers, in which deregulation of Wnt signaling is a major initiation step, and of other diseases, such as familial osteoporosis. Recognizing the importance and rapid development of this field, the Juan March Foundation sponsored a workshop on "*wnt* genes and Wnt signaling," which was organized by Roel Nusse (Stanford University), Jose Felix de Celis (Centro de Biologia Molecular, Madrid), and Juan Carlos Izpisua Belmonte (Salk Institute, La Jolla) and took place in Madrid, March 24–26, 2003. While the meeting highlighted a broad range of roles for Wnt signaling in development, including early axis specification (Huelsken et al., 2000), head induction (Mukhopadhyay et al., 2001), brain patterning (Heisenberg et al., 2001; Houart et al., 2002), mesenchymal-epithelial interaction (Kratochwil et al., 2002), chondrogenesis (Hartmann and Tabin, 2000, 2001), and intestinal development (Batlle et al., 2002; van de Wetering et al., 2002), here I will, because of space constraints, focus on discussions of the implications that these and other studies have had for our understanding of how Wnt signaling pathways operate.

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Canonical Wnt/β-Catenin Signaling

Wnt signaling through β-catenin is most intensively studied and, as expected, was the most discussed topic of the meeting. The prevailing view of β-catenin function in this pathway is that it associates with, and acts as an obligatory coactivator for, the TCF/LEF (T cell factor/lymphoid enhancer factor) family of transcription factors (Bienz and Clevers, 2003). In the absence of a Wnt ligand, the level of cytosolic β-catenin is kept low because of phosphorylation-dependent ubiquitination/proteosome degradation; thus, TCF/LEF is associated with transcription corepressors and suppresses Wnt-responsive gene expression (Figure 1A). Upon Wnt stimulation, β-catenin phosphorylation and subsequent degradation is prevented, and the resulting accumulation of β-catenin promotes its association with TCF/LEF, thereby activating Wnt-responsive transcription (Figure 1B). According to this model, β-catenin phosphorylation is a critical step in controlling its abundance. Indeed, this involves the sequential actions of casein kinase I (CKI) and glycogen synthase kinase 3 (GSK-3) and takes place in a protein complex assembled by the scaffolding protein Axin and the tumor suppressor protein APC, the *adenomatous polyposis coli* gene product (Figure 1A). Any perturbation of this process, such as a loss of functional APC (and sometimes Axin) or β-catenin mutations that prevent its phosphorylation/degradation, will cause constitutive β-catenin signaling that is believed to be a key molecular underpinning for colorectal tumorigenesis. This complex β-catenin phosphorylation/degradation, together with other potential regulatory mechanisms, such as cytoplasmic/nuclear anchoring/shuttling of β-catenin, invites regulatory inputs at many levels.

Wnt Receptors

Frizzled (Fz) serpentine receptors have been established as Wnt receptors and can bind Wnt proteins with a K_d of 1.6 nM (R. Nusse). Rigorous genetic and biochemical evidence for ligand-receptor relationship between Wnt and Fz, however, has thus far been demonstrated only for Wingless (Wg) and *Drosophila* Frizzled (DFz1) and DFz2. A lack of purified Wnt proteins poses problems for Wnt-Fz binding studies, and the large number of Wnt and Fz proteins (we have 19 Wnts and 10 Fzs in our genome) and the likelihood of a multitude of Wnt-Fz interactions—one Wnt may bind several Fzs, and one Fz may bind several Wnts—complicate the issue. A newcomer to Wnt reception is a single-pass transmembrane receptor, *Drosophila* Arrow/vertebrate LRP5 and LRP6 (LDL receptor-related protein). One model, based on in vitro binding results, suggests that Wnt might induce Fz and LRP5/6 to form a receptor complex (reviewed in Zorn, 2001). A recent demonstration of the constitutive activity of DFz2 fused to the intracellular domain of Arrow is consistent with this model (Tolwinski et al., 2003). Extracellular domain-deleted LRP5/6 and Arrow mutants trigger constitutive β-catenin signaling in a number of assays (Mao et al., 2001a, 2001b; Xi He, Children's Hospital/Harvard Medical School; Keith Brannan, University of Manchester, UK; Anthony Brown, Weill Medical College of Cornell University, NY), apparently in

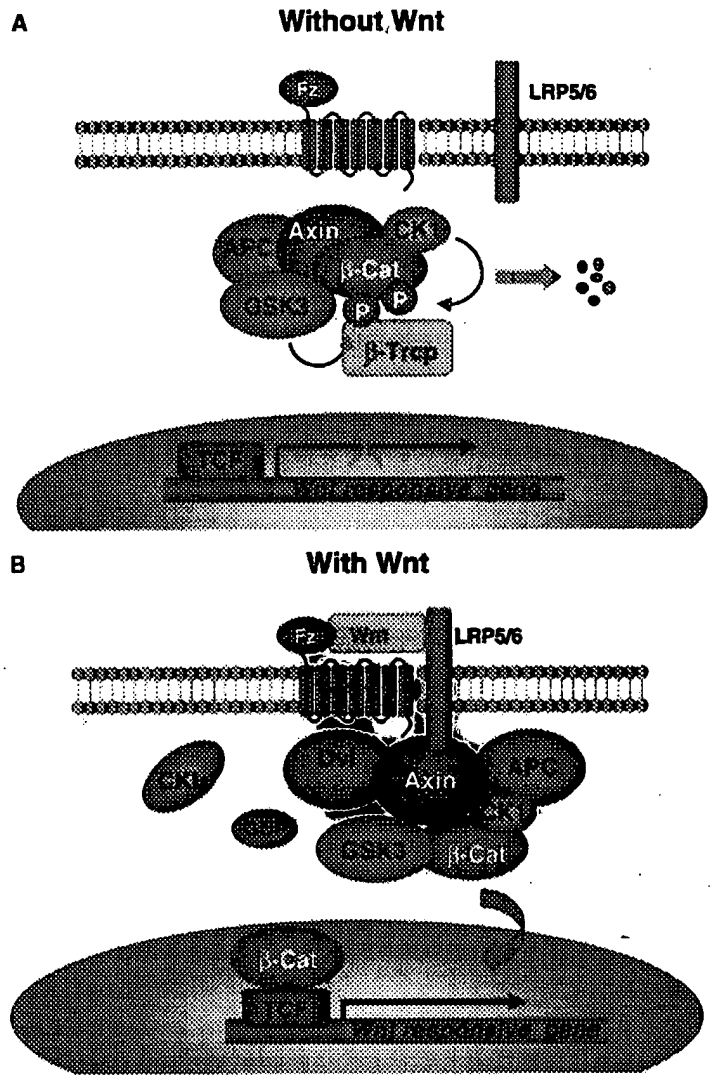

Figure 1. A Working Model for the Wnt/β-Catenin Signaling Pathway

Readers may refer to http://www.stanford.edu/~musse/wntwindow.html for more detailed descriptions of various components.

(A) Without Wnt, β-catenin is phosphorylated in the Axin complex by CKI and then by GSK-3, and the phsophorylated β-catenin is recognized by β-Trcp for subsequent ubiquitination and degradation. TCF suppresses gene transcription. Other components in the Axin complex, such as PP2A and Diversin, and TCF-associated corepressors are omitted for simplicity.

(B) Wnt stimulation resulting in a Dvl-dependent inhibition of the Axin complex and, thus, of β-catenin phosphorylation/degradation. Accumulated β-catenin forms a complex with TCF and activates transcription. This may involve Wnt-induced complex formation between Fz and LRP5/6, which may recruit Axin to the plasma membrane. The composition of the Axin complex in Wnt-stimulated cells is not well defined. CKIε can activate β-catenin signaling by an unknown mechanism. β-catenin-associated coactivators are omitted for simplicity.

a Wnt- and Fz-independent manner. How Arrow/LRP5/6 is activated by Wnt signaling is unknown.

LRP5/6 activity is regulated by several ligands in addition to Wnts. One of these is Dickkopf-1 (Dkk-1), which is a vertebrate head inducer and antagonizes LRP5/6 and Wnt/β-catenin signaling (Zorn, 2001). Paradoxically, Dkk-2 can activate or inhibit LRP5/6 function in different cell types or assays (Christof Niehrs, Deutsches Krebsforschungszentrum, Heidelberg; Mao and Niehrs, 2003). Nobue Itasaki (National Institute for Medical Research, London) identified another secreted molecule, called WISE, which also exhibits such dual activities. In *Xenopus* embryos, WISE can activate, albeit moderately, β-catenin target genes *Xnr3* and *siamois*, and increase β-catenin nuclear localization. However, when combined with a BMP (bone morphogenetic protein) inhibitor, WISE, like Dkk-1, acts like a Wnt inhibitor in head induction. WISE binds to LRP6 and blocks Xwnt-8 binding to LRP6. Niehrs discussed regulation of LRP6 function by another family of Dkk-1 receptors, Kremen1 and -2 (Krm) (Mao et al., 2002). Krms are single transmembrane proteins that synergize with Dkk-1 to inhibit LRP6 function. Krms can form a complex with LRP6 and Dkk-1 and, upon overexpression, cause internalization of LRP6 from the cell surface. This model—that Dkk-1 via Krm promotes LRP6 internalization—is different, although not mutually exclusive, from a previous model in which Dkk-1 disrupts Fz-LRP6 complex formation (Semenov et al., 2001). Notably Dkks, Krms, and WISE, like other secreted Wnt antagonists, are thus far found and conserved only in vertebrates.

The Axin Complex, Dishevelled, and "Vesicles"?

It appears that Wnt activation of Arrow/LRP5/6 recruits Axin to the plasma membrane (Mao et al., 2001b; Tolwinski et al., 2003). Mariann Bienz (MRC, Cambridge) has provided further evidence for this model using the Axin-GFP (green fluorescence protein) fusion protein in *Drosophila* embryos. She and her colleagues found that Axin-GFP is present in the cytoplasm as "dots" but becomes concentrated near the plasma membrane in cells

where Wg signaling is active, and overexpressi n f Wg increases Axin-GFP localization t the plasma membrane (Cliffe et al., 2003). Immunostaining for on f the two *Drosophila* APC pr teins, E-APC, shows colocalization with the cytoplasmic Axin-GFP dots, which disappear and are replaced by diffuse Axin-GFP staining in the cytosol in cells lacking APC function (Cliffe et al., 2003).

The cytoplasmic protein Dishevelled (Dsh in *Drosophila*; Dvl1-3 in mice) is an essential, but mysterious, component in Wnt signaling and functions epistatically downstream of Wnt receptors, but upstream of Axin (Tolwinski et al., 2003). Indeed, Dsh (but neither GSK-3 nor APC) is required for Axin-GFP plasma membrane localization triggered by Wg signaling. Dsh-GFP itself shows plasma membrane localization (which does not appear to change during Wg signaling) and also appears in some intracellular dots (Cliffe et al., 2003). Bienz suggested that, in response to Wg, Dsh might shuttle the Axin complex to the plasma membrane via a yet to be defined vesicular transport mechanism. This view was echoed by a recent paper (Capelluto et al., 2002) showing that the DIX domain of Dvl2 binds phospholipids and that this activity is required for Dvl2 vesicle-like localization and its ability to activate β-catenin signaling. These results imply that Dvl association with vesicular membranes is required for Wnt/β-catenin signaling. Consistent with this view, Elaine Seto (Baylor College of Medicine, Houston) showed that certain components involved in membrane trafficking, such as specific Rab GTPases, are required for Wg response during fly wing development. A cautionary note is warranted, however, since no direct evidence exists yet for these hypothetical vesicles.

The vesicular transport issue was also discussed in a talk by Rik Korswagen (Netherlands Institute of Developmental Biology, Utrecht) on Wnt regulation of Q neuroblast migration in *C. elegans*. Although the two Q cells, QL (left) and QR (right), are born at similar positions and generate an identical set of descendants, QL and descendants express a homeobox gene, *mab-5*, and migrate posteriorly, whereas QR and descendants do not express *mab-5* and migrate anteriorly. EGL-20/Wnt activates *mab-5* expression in QL cells via the canonical β-catenin pathway (Korswagen et al., 2002). Using a genomewide RNAi screen for genes affecting Q cell migration, Korswagen's laboratory identified 11 genes, including a homolog of Vps35, a component of the yeast retromer complex involved in vesicular recycling between late endosome compartments and the Golgi apparatus. A *vps-35* null mutant phenocopies the *egl-20/wnt* mutant, and epistasis analysis positions VPS-35 upstream of PRY-1/Axin. It will be critical to resolve whether VPS-35 acts in EGL-20/Wnt-producing or QL and QR (responding) cells.

Who and How Does Wnt regulate: Axin, GSK-3, and/or CKI?

GSK-3 has long be n a leading suspect for mediating Wnt regulation. Indeed, GSK-3 ph sphorylation of β-catenin is inhibited upon Wnt signaling (Liu et al., 2002). GBP (GSK-3 binding prot in)/Frat, which binds t both Dvl and GSK-3, is a candidate for mediating this r gulation. Trevor Dal (Institut of Canc r R search, L nd n) discussed two aspects of GSK-3 regulation by Frat. Frat may compete GSK-3 away from the Axin c mplex (Dajani t al., 2003); it may also rem ve GSK-3 from nucl i (Franca-K h t al., 2002). Dale proposed that nuclear GSK-3 might phosphorylate APC, which, in turn, exhibits higher affinity for β-catenin for mor efficient export of β-catenin (Rosin-Arbesfeld et al., 2000). However, it remains unclear whether Frat/GBP plays a key role in Wnt signaling, as Frat/GBP homologs have not been found in invertebrates, and a Dvl1 mutant lacking the Frat binding domain still activates β-catenin signaling (Hino et al., 2003).

Despite the observation that GSK-3 phosphorylation of β-catenin is stimulated 20,000-fold in the presence of Axin (Dajani et al., 2003), CKI is nonetheless required as a priming kinase in vivo (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). While most agree that CKIα primes β-catenin phosphorylation at serine 45 (S45), whether CKIε also functions here is a subject of debate. CKIε, like CKIα, is present in the Axin complex and was initially found, by a still obscure mechanism, to stimulate Wnt/β-catenin signaling (reviewed in Polakis, 2002). Yinon Ben-Neriah (Hebrew University, Jerusalem) presented data showing that whether CKIε phosphorylates β-catenin S45 depends on the cell types examined. siRNA against CKIα, but not CKIε, decreases S45 phosphorylation in HeLa cells, whereas, in RKO cells, siRNA against either CKIα or CKIε inhibits S45 phosphorylation, analogous to what was observed in *Drosophila* S2 cells (Yanagawa et al., 2002). These observations also raise the question of whether Wnt regulates β-catenin phosphorylation by GSK-3 (at threonine 41, S37, and S33) or by CKI at S45. Ben-Neriah showed that Wnt-3a and Dvl can both inhibit S45 phosphorylation (Amit et al., 2002), but experiments with Wnt-1 failed to demonstrate a similar effect (Liu et al., 2002).

Given that CKI and GSK-3 phosphorylation of β-catenin occur within the Axin complex and that Axin exists in rate-limiting amounts, Wnt-induced Axin degradation (Yamamoto et al., 1999; Willert et al., 1999) may be important for β-catenin signaling. A recent report, showing that, in *Drosophila* embryos, Wg signaling reduces the level of overexpressed Axin protein, argues for this possibility (Tolwinski et al., 2003). However, Bienz laboratory's experiments in fly embryos did not record much effect of Wg signaling on Axin-GFP protein level (Cliffe et al., 2003). One explanation is that the GFP moiety may stabilize Axin-GFP. On the other hand, it needs to be determined whether Wg reduction of Axin protein level seen by Tolwinski et al. is a primary event. Indeed, although prolonged Wnt-3a treatment of mammalian cells decreases Axin protein level, Wnt-3a-induced β-catenin accumulation at early time points of Wnt treatment occurs without obvious reduction of endogenous Axin protein level (Willert et al., 1999).

Attendees also discussed a result of Tolwinski et al. that a weakly functional Armadillo (β-catenin) is regulated by Wg in fly embryos that are mutant f r zw3 (GSK-3), but n t in embry s mutant for Axin. This impli s a Wnt pathway that inhibits Axin, but bypasses GSK-3, at least in cells with a w akly active β-catenin. This GSK-3-independent Axin function may be, in part, attributed to its cytoplasmic ret nti n of β-catenin (T lwinski

and Wieschaus, 2001), but it remains a question whether th ther GSK-3-like kinase in the fly g nome (M rrison et al., 2000) partially compensates for ZW3 und r this c ndition. Of not , th zebrafish *masterblind* mutation, which exhibits hyperactive Wnt/β-catenin signaling, is a result of a single-amino acid change in Axin1 that abolishes Axin1-GSK-3 interaction (Stephen Wilson, University College London, UK; Heisenberg et al., 2001; van de Water et al., 2001). Dale noted a converse observation that overexpression of a GSK-3 mutant that cannot bind Axin nonetheless inhibits TCF/β-catenin-dependent reporter expression. These results together may hint at the existence of multiple parallel mechanisms for regulating β-catenin abundance, cytoplasmic/nuclear partitioning, or activity, thereby ensuring tight regulation of Wnt/β-catenin signaling.

β-*Catenin and TCF/LEF*

Although a debate over whether β-catenin has mainly a cytoplasmic or nuclear role in TCF/LEF-dependent gene expression was reignited (Chan and Struhl, 2002), the prevailing view remains that β-catenin functions as a coactivator for TCF/LEF (reviewed in Bienz and Clevers, 2003). The argument for a cytoplasmic role of β-catenin, derived from using membrane-tethered forms of β-catenin, is complicated by the difficulty to create a null background of β-catenin/Armadillo (Chan and Struhl, 2002).

Rudolf Grosschedl (University of Munich, Germany) created knockin mice in which an endogenous *lef1* allele was replaced with *lef1*^{m5}, which encodes a mutant LEF1 with six amino acid substitutions in its β-catenin binding region. *lef1*$^{m5/-}$ mice exhibit indistinguishable phenotypes from *lef1*$^{-/-}$ null mice, such as defective development of neural crest cells, whisker follicles, teeth, and the hippocampus, demonstrating that LEF1 function in vivo depends on its association with β-catenin. However, LEF1 may have a β-catenin-independent function in activation of the T cell receptor-α gene, but this function is redundant with TCF1 and is thus precluded from analysis.

The TCF/LEF-β-catenin complex in the nucleus activates transcription via recruiting other cofactors, including those commonly required, such as p300/CBP, and those specifically devoted to the Wnt/β-catenin pathway, such as Pygopus (Pygo) (Ken Cadigan, University of Michgan, Ann Arbor; Kramps et al., 2002; Thompson et al., 2002; Parker et al., 2002; Belenkaya et al., 2002) and Legless (Lgs)/Bcl9. Lgs is the *Drosophila* homolog of mammalian Bcl9 oncoprotein and is suggested by genetic and biochemical analyses to be an adaptor protein linking β-catenin and Pygo (Kramps et al., 2002). Felix Brembeck and Walter Birchmeier (Max Delbrueck Center, Berlin) isolated another Lgs homolog, Bcl9.2, as a β-catenin binding protein. In zebrafish embryos, depletion of Bcl9.2 via a morpholino antisense oligo prevents trunk/tail development and, thus, phenotypically copies mutant embryos lacking zygotic Wnt-8 activity (Lekven et al., 2001).

Akira Kikuchi (Hiroshima University, Japan) pres nted data on regulati n f TCF4 activity by sumoylation (Yamamoto et al., 2003). Like LEF1, TCF4 is sum ylated by the sum -ligase PIASy (Sachdev et al., 2001). TCF4, when overexpressed alone, exhibits diffuse nucl ar staining, but PIASy coexpr ssion caus s TCF4 to relocalize to "nuclear bodies," where PIASy and PML are colocalized. Although Axam, a pr tein with a desum y-lati n motif, was identified by Kikuchi's group as an Axin binding protein and may have activities that regulate β-catenin stability (Kad ya t al., 2002), Axam also plays a role in desumoylation of TCF4, as revealed by siRNA against Axam. Like LEF1 sumoylation, TCF4 sumoylation has no effect on TCF4 DNA or β-catenin binding. However, TCF4 sumoylation increases, whereas LEF1 sumoylation may decrease, transcriptional activation (Sachdev et al., 2001; Yamamoto et al., 2003).

Wnt/β-catenin signaling controls many developmental decisions, yet how different tissue- or stage-specific responses are achieved is not well understood. One example of how specificity is achieved at the transcriptional level is seen in early *Xenopus* development. Maternal β-catenin signaling promotes dorsal axis formation, whereas zygotic Xwnt-8/β-catenin signaling shortly after is required for ventrolateral development. Stefan Hoppler (University of Dundee, Scotland) presented evidence that TCF3 and LEF1 are required for maternal and zygotic β-catenin signaling, respectively (Roel et al., 2003). Rolf Kemler (Max Planck Institute for Immunology, Freiberg, Germany) discussed yet another strategy, in which other transcription factors, like Pitx2, may serve as determinants of target specificity (Kioussi et al., 2002). Interestingly, the *pitx2* gene is induced by earlier TCF/β-catenin signaling. Cadigan described a *Drosophila* gene called *split ends* (*spen*), which has specific roles during Wg signaling in eye, wing, and leg development, but not in early embryos (Lin et al., 2003). The *spen* gene encodes a large nuclear protein with RNA recognition motifs, and its human homolog, SHARP, is implicated as a corepressor for nuclear receptors.

Feedback Regulation and Crosstalk

Wnt/β-catenin signaling induces, directly or indirectly, genes whose products are involved in Wnt signaling or feedback regulation. Examples include Wg suppression of *DFz2* and *arrow* genes, induction of the *naked cuticle* gene, and Wnt induction of *axin2/conductin*, β-*Trcp* (a ubiquitin-ligase for β-catenin), and secreted Wnt antagonist genes. Keith Wharton (UT Southwestern Medical Center, Dallas) discussed the *Drosophila* Naked cuticle (Nkd) protein and its vertebrate homologs, which are EF hand-containing proteins and bind to and antagonize Dsh/Dvl via binding to the Dsh/Dvl PDZ domain (Wharton et al., 2001). The *axin2/conductin* gene is a direct target gene induced by the TCF/β-catenin complex and thus feeds back to inhibit Wnt signaling and shows oscillating expression during somite formation. This negative-feedback loop may underlie Wnt-3a regulation of the segmentation clock in chick and mice (R. Kemler; Aulehla et al., 2003).

Wnt and Notch signaling crossregulate many developmental events and often exhibit complex genetic interactions. Alfonso Martinez Arias (University of Cambridge, UK) described a branch of Notch signaling, which is independent of Su(H) (suppressor of hairless) and antagoniz s Wg signaling. He noted that overexpressi n of Armadillo or TCF al ne or in combination in *Drosophila* wing does not l ad to Wg signaling, whereas loss of Notch function in some cases results in Dsh-independent Wg signaling (Martinez Arias et al., 2002). Thus, TCF and/or β-catenin activity may subject to additional regulation, such as by N tch signaling. Although

Dsh can bind to the intracellular domain f Notch (Axelrod et al., 1996), the mechanism of th interaction between Notch and Wnt signaling remains obscure.

Noncanonical Wnt/Fz Signaling

Wnt Signaling in Axonal Morphogenesis

Wnt signaling regulates not only neural patterning and fate, but also axonal growth, path finding, and synapse formation. Patricia Salinas (Imperial College, London) described two cases studied in her lab: Wnt-7a regulation of mossy fiber synaptogenesis in cerebellum and Wnt-3 regulation of terminal arborization of sensory neurons in spinal cord (Krylova et al., 2002). These Wnts from target cells (granule cells or motor neurons) promote incoming connecting axons to form branches, increase growth cone size, and stop axonal extension. A key aspect of this Wnt regulation is mediated via Dvl and GSK-3 action on microtubule (MT) stability and is independent of β-catenin, TCF, or transcription in general. Dvl via its PDZ domain binds to, and stabilizes, MTs. GSK-3 antagonizes Dvl stabilization of MTs. GSK-3 can directly phosphorylate MT-associated protein MAP-1B, and this phosphorylation appears to promote the dynamic instability of MTs (Lucas et al., 1998). Dvl downregulates MAP-1B phosphorylation by GSK-3, but how Dvl inhibits GSK-3 is unknown; GBP/Frat does not appear to be involved.

Fz Planar Cell Polarity (PCP) Signaling

Peter Lawrence (MRC, Cambridge) described Fz- and Dsh-dependent PCP signaling in the *Drosophila* adult abdomen. A morphogen gradient ("X") has long been hypothesized to initiate Fz PCP signaling, but the existence/identity of X remains elusive. Since neither the deficiency of five of the seven *Drosophila wnt* genes nor overexpression of any of them produces consistent PCP perturbation (Lawrence et al., 2002), X may not be a Wnt. The situation is different in vertebrates, however, as studies in *Xenopus* and zebrafish suggest that Wnt-11/Silberblick activates a pathway similar to Fz/PCP signaling in the regulation of convergent extension movements during gastrulation (Tada and Smith, 2000; Heisenberg et al., 2000). This Wnt/Fz pathway appears to employ Dvl to stimulate RhoA/ROK and Rac/JNK activities and is likely mediated by independent Dvl-RhoA and Dvl-Rac complexes induced upon Wnt signaling (Habas et al., 2003). Masazumi Tada (University College London, UK) showed that this pathway also includes zebrafish *prickle1* (*pk1*), which is the homolog of the *Drosophila prickle* gene implicated in Fz/PCP signaling (see also Veeman et al., 2003; Takeuchi et al., 2003).

A Purified Wnt Molecule (Finally)

Our advances in understanding Wnt function and signaling, ironically, are accompanied by little progress in understanding Wnt proteins, largely because of failure to purify them despite years of painstaking effort. Nusse described the purification of mouse Wnt-3a and *Drosophila* Wnt-8 via sequential chromatography (Will rt et al., 2003), thus putting this part of the Wnt history b hind us. Purified Wnt-3a prot in is functional and unequivocally shows that n ither additi nal posttranslational cleavage (other than the signal peptide cl avage) nor any c factor is required for Wnt function. Most inter stingly, Wnt-3a is palmitoylated at cysteine 77, which corresponds to the first of 22 invariable cysteine residues cons rved among all Wnt proteins. Purified Wnt-3a treated with an acyl-protein thioesterase, which removes the palmitate, or the Wnt-3a(C77A) mutant that cannot be palmitoylated, loses significant bioactivity in β-catenin stabilization assays. Additionally, a temperature-sensitive allele, wg^{ts21} (isolated by Martinez Arias), and an apparent null allele, egl-20 (N585), are missense mutations of the equivalent cysteine residues in *Drosophila* Wg (C93) and *C. elegans* EGL-20 (C99), respectively. However, Wnt-3a(C77A), upon overexpression, can modestly induce β-catenin level in transfected cells, and Wg^{ts21} at permissive temperatures can signal to cells in close range, suggesting that Wnt palmitoylation is not absolutely required for signaling. Whether this modification affects Wnt secretion, transport, or receptor binding remains to be resolved. Nusse speculated that the *Drosophila porcupine* and nematode *mom-1* (and by extension their vertebrate homologs), which are required in Wg/Wnt-secreting cells and encode apparent acyltransferases, might be responsible for Wnt palmitoylation. The purification of Wnt proteins opens many opportunities, including the examination of Wnt-receptor relationships in biochemical detail.

The Future

The multitude of Wnt functions and the complexity of Wnt signaling are just beginning to be elucidated. We need a better understanding of how Wnt proteins are produced and secreted and form morphogen gradients, how they engage various receptors, such as Fz, LRP, and a recently described atypical tyrosine kinase receptor (Yoshikawa et al., 2003), and how these receptors are activated and interact to specify activation of different Wnt pathways. We still know very little about how receptor activation initiates intracellular signaling, how various components, including Dvl and the Axin complex, are regulated, and how β-catenin activity, in addition to its abundance, is governed. We want to learn more about noncanonical Wnt signaling pathways, including PCP/Rho/Rac, Ca^{2+}/PKC, and those mediating Wnt regulation of axon growth, guidance, and synaptogenesis. As more and more Wnt functions are being revealed in different model systems, we will have to address how Wnt signaling, via a limited number of pathways, executes a vast variety of developmental programs. At least some of these are mediated by distinct genomic responses to Wnt signaling, as demonstrated by comprehensive gene profiling studies during mouse axis and mesoderm formation (W. Birchmeier), T cell development (R. Grosschedl), and intestinal development and colorectal tumorigenesis (Hans Clevers, Netherlands Institute of Developmental Biology, Utrecht; Batlle et al., 2002; van de Wetering et al., 2002). Furthermore, these genomic responses involve not only genes encoding prot ins, but als others, such as those encoding microRNAs, as exemplified by the *Drosophila bantam* gene that regulates both proliferati n and apopt sis (Steve Cohen, EMBL, H idelberg; Brennecke et al., 2003). Answ rs to many of the ab v questions will undoubtedly be helped by new technologies, including

genomics and proteomics. As Wnt molecules c ntrol stem c ll renewal during h mat poi sis (R. Nusse; Willert et al., 2003) and intestinal developm nt (H. Cl v rs; van de Wet ring et al., 2002), and defectiv Wnt signaling is involved in human cancers and diseases, basic Wnt research will find applications in clinical studies, from pathology to therapeutics. When developmental biologists and cancer researchers get together, such as at this Juan March workshop, although it may not always be a "Wnt-Wnt" situation, it will always be a win-win situation.

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Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15

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Most mammary carcinomas induced in C3H mice by the mouse mammary tumour virus (MMTV) bear a new proviral insertion within a highly conserved locus on chromosome 15 called int-*1. A transcriptional unit within this locus is inactive in all tested normal tissues but expressed at low levels in mammary tumours with proviral insertions positioned on either the 5′ and 3′ sides of the gene. Transcription of the proviruses proceeds away from* int-*1; thus an indirect mechanism appears to activate expression of this putative oncogene.*

THE proviruses of retroviruses can integrate at many sites in host genomes; thus, like other movable genetic elements, they act as insertion mutagens[1]. Two classes of proviral insertion mutations have been described: recessive mutations that disrupt and prevent expression of genes[2-6] and presumably dominant mutations that activate gene expression, leading to induction of tumours by retroviruses without their own oncogenes[7-13]. For example, during the induction of B-cell lymphoma by the avian leukosis virus (ALV), proviral insertion mutations augment transcription of c-*myc*, the cellular progenitor of a retroviral onc gene[7], either by provision of a retroviral promoter[7-10] or by an indirect effect on a host promoter[10].

The proposal that retroviruses lacking oncogenes induce tumours by insertion mutation of host genes suggests a strategy for the isolation of novel oncogenes. By molecular cloning of a single new mouse mammary tumour virus (MMTV) provirus from a C3H mouse mammary tumour, we have isolated a region of the mouse genome that contains an MMTV proviral insertion in the majority of C3H mammary tumours[13]. Although this region lacks homology with any of the available retroviral oncogenes, we have previously argued that it harbours an oncogene, called *int*-1, instrumental in mammary tumorigenesis because (1) MMTV proviruses can integrate at many sites in the host genome (implying that insertions near *int*-1 confer a selective growth advantage) and (2) because the insertions are accompanied by the appearance of transcriptional products from *int*-1, a silent domain in non-neoplastic mammary tissue.

We demonstrate here that insertion mutations in the *int*-1 locus usually stimulate transcriptional activity by an indirect mechanism: MMTV proviruses are located either upstream from transcribed *int*-1 sequences in the opposite orientation or downstream in the same orientation. We also assign the *int*-1 gene to mouse chromosome 15 and show that *int*-1, like the progenitors of viral oncogenes, has been conserved during evolution.

Locating and orienting MMTV proviruses

We reported previously[13] that 18 of 26 C3H mouse mammary tumours contain MMTV proviral DNA within a common region of approximately 20 kilobases (kb). The insertions are distributed with roughly equal frequency on both sides of a portion of the locus that is represented in tumour-specific, 2.6-kb polyadenylated RNA. To assess the mechanisms involved in the activation of the *int*-1 transcriptional unit, we have now determined the sites of proviral insertion more precisely and ascertained the transcriptional directions of the proviruses and the *int*-1 gene. These studies involved physical mapping of the interrupted *int*-1 loci with restriction endonucleases, using the DNA transfer method and a collection of molecular annealing reagents specific for portions of *int*-1 and for the 5′ and 3′ halves of MMTV proviral DNA. The *int*-1 probes were derived by plasmid subcloning of unique sequence DNA from recombinant λ bacteriophages containing overlapping inserts of the *int*-1 domain from a library of normal BALB/c mouse DNA. Probe C detects *int*-1 RNA in mammary tumours; probe D represents a region located leftward of probe C on the *int*-1 map as it is conventionally drawn (see Fig. 3). The probes used to orientate proviral DNA [MMTV-*env* and MMTV-*gag* (Fig. 1)] represent portions of MMTV DNA that reside near the 5′ and 3′ ends of the provirus but exclude sequences from the long terminal repeats (LTRs). In general, our strategy was to use enzymes that cleaved *int*-1 at widely separated sites and divided MMTV proviral DNA into two portions distinguishable with MMTV-*env* and MMTV-*gag* probes.

Proviruses on the 5′ side of *int*-1

Two enzymes, *Bgl*II and *Kpn*I, proved to be particularly useful for the analysis of MMTV proviruses present to the left of the *int*-1 transcriptional unit. (We will show below that transcription of *int*-1 proceeds from left to right on the physical map; thus, proviruses inserted to the left of the region represented by probe C can be considered to lie 5′ to at least a portion of the transcriptional template.) *Bgl*II cleaves the normal C3H mouse DNA at a leftward site (position −21 on the map in Fig. 3) to generate a 14-kb fragment that anneals with probe C. In addition, *Bgl*II cleaves the MMTV provirus at two sites, one of them near the middle of the element (Fig. 1). Proviral DNA in eight tumours was found to disrupt the normal 14-kb *Bgl*II fragment from one of the two *int*-1 alleles. As illustrated for six of these tumours in Fig. 1*a* and *b*, the novel fragments also annealed with the MMTV-*gag* probe, indicating that the proviruses are positioned either to the right of the probe C homology in the same transcriptional orientation as *int*-1 or to the left of the probe C region in the opposite orientation. In all eight tumours, additional mapping, illustrated here for a few tumours with *Kpn*I and probe D, showed that the latter possibility was correct (Fig. 1*c*, *d*). *Kpn*I cleaves the *int*-1 domain at the most leftward site we have mapped (position −23) and at position −11, to generate a fragment of 12 kb that hybridizes with probe D. The MMTV provirus is cut once by *Kpn*I. Novel *Kpn*I fragments annealing with probe D (Fig. 1*c*) were also detected by the MMTV-*env* probe (Fig. 1*d*), demonstrating that the proviruses are inserted upstream from the probe C homology region in orientations opposite to the transcriptional direction of *int*-1.

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Table 1 Segregation of mouse *int*-1 and mouse chromosome in Chinese hamster–mouse somatic cell hybrids

Chromosome	Marker enzyme	*Int*-1/chromosome (no. of clones) +/+	+/−	−/+	−/−	% Discordance
1	PEP-3	5	4	1	2	42
2	AK-1	15	2	8	4	34
3	—	2	7	1	2	67
4	PGD	13	5	4	8	30
5	—	2	7	1	2	67
6	TPI-1	3	6	7	5	62
7	GPI-1	4	5	2	1	58
8	GR-1	4	5	1	2	50
9	MOD-1	1	8	1	2	75
10	PEP-2	2	7	2	1	75
11	—	0	9	0	3	75
12	ACP-1	5	4	1	2	42
13	—	4	5	0	3	42
14	NP-1	10	6	5	7	39
15	AS-2	17	0	1	10	4
16	SOD-1	3	6	2	0	73
17	GLO-1	2	7	1	2	67
18	PEP-1	7	2	5	8	32
19	GOT-1	12	6	1	11	23
X	HPRT	15	3	12	0	50
Y	—	0	9	0	3	75

Thirty clones of hybrids formed by polyethylene glycol fusion of mouse spleen cells or macrophages with an established Chinese hamster cell line (380-6), deficient for hypoxanthine phosphoribosyl transferase (HPRT), were analysed for mouse *int*-1 and enzyme markers, although not all clones were analysed for every enzyme marker. Twelve of the hybrid clones were also analysed by karyotype analysis as decribed previously[22]. The symbols for the marker enzymes, their chromosome assignments and the electrophoretic procedures used to separate the Chinese hamster and mouse enzymes have been previously described[22]. In general, the presence or absence of a given mouse chromosome, as determined by karyotypic analysis, agreed with the presence or absence, respectively, of the enzyme marker for that chromosome. However, in several instances, the enzyme marker for a given chromosome was present in a clone that karyotypically lacked that mouse chromosome, indicating chromosome breakage and/or rearrangement. The presence of mouse chromosomes 3, 5, 11, 13 and Y was based on karyotype analysis alone.

Proviruses on the 3′ side of *int*-1

Similar analytical strategies were applied to DNA from 10 tumours previously shown to have insertions between positions 0 and −8 on the *int*-1 map; an 11th tumour (number 24), previously thought to lack an *int*-1 insertion, was also found to have a provirus within this region during the extended tests. Comparative annealings with *int*-1 and MMTV probes (ref. 13 and data not shown) revealed that all 11 proviruses were oriented in the same direction, with transcription proceeding from left to right on the *int*-1 map.

A particularly interesting part of this analysis, pertinent to the mechanism of expression of *int*-1, is illustrated in Fig. 2. When DNA from tumour 53 was cleaved with *Bam*HI and annealed with probe C, two novel fragments were detected in digests of each sample, indicating that the insert is positioned within the region detected by the probe. Similar results were obtained with DNA from tumour 11 (not shown). Proviruses in three other tumours (numbers 55, 12 and 15) were found to be positioned at very similar sites, about 0.6–0.8 kb to the right of the insertion site in tumour 53 (Fig. 2 and unpublished data). The inserts in these tumours lie within the transcriptional unit, and affect the size of resulting transcripts (see below).

We conclude that 19 of 26 C3H mammary tumours carry MMTV proviruses in the *int*-1 locus (Fig. 3). Eight proviruses are distributed between positions −11 and −18; each of these is oriented so that transcription of the provirus proceeds from right to left. Eleven proviruses are located within the region extending from position −8 to 0, and all are oriented so that transcription proceeds from left to right. Mapping with several end nucleases provides no evidence for deletions within either proviral DNA or *int*-1; the several atypical restriction maps of proviral DNA we encountered are best explained by restriction site polymorphisms (data not shown).

int-1 transcripts

We have previously shown that tumours bearing proviruses in the *int*-1 region contain polyadenylated transcripts that anneal with probe C, whereas no RNA homologous to probe C is detectable in normal mammary glands[13]. The abundance of the *int*-1 transcripts is not great; less than 10 copies per cell have been estimated by three independent methods (unpublished results).

To judge the mechanism by which the insertion mutations activate expression of *int*-1, it was necessary to determine the direction of transcription of the cellular gene. The *Bam*HI–*Eco*RI fragment that constitutes probe C was recloned in both orientations in the bacteriophage vector M13. Restriction mapping of the replicative forms established the orientation of each insert and hence the polarity of strands present in single-stranded virion DNA (data not shown). Radioactive probes were synthesized by random priming on templates provided by two phages, each bearing one of the two strands. Only one of these probes was found to anneal to RNA from tumour 44, previously shown to contain an *int*-1 transcript 2.6 kb in length (Fig. 4*a*), the composition of the probe established the direction of transcription to be from left to right on the *int*-1 map as presented in Fig. 3.

The *int*-1 transcripts characterized in our earlier report were, like those in tumour 44, approximately 2.6 kb long[13]. In a wider survey of all those tumours from which undegraded polyadenylated RNA could be obtained, we discovered atypical *int*-1 transcripts of 3.2 kb in tumour 11 and of 3.8 kb in tumours 12 and 15 (Fig. 4*b*). In these three tumours, no 2.6-kb *int*-1 RNA was found. The conventional 2.6 kb *int*-1 RNA species was present in all the other tested tumours with *int*-1 insertions (for example, in tumours 24 and 13 in Fig. 4*b*) and in one tumour (number 14) in which an *int*-1 mutation has not been found (unpublished data).

The probable composition of the larger, atypical RNA species was suggested by an intriguing correlation with the site of insertion: in tumours 11, 12 and 15, integration of proviral DNA occurred within or near the right-hand boundary of the region represented by probe C (see Fig. 2). (Suitable RNA samples were not available from the other tumours, 53 and 55, with insertions in this region.) If the proviruses were located within the transcribed domain, upstream from the natural polyadenylation site for *int*-1 RNA, the 5′ LTR would be appropriately positioned to provide a surrogate polyadenylation site. The enlarged size of the *int*-1 transcript could then be ascribed to the length of the U3 region of the MMTV LTR (~1.2 kb)[14–16]. A similar situation has been encountered in an avian leukosis virus (ALV) insertion mutant affecting the chicken c-*myc* locus[10].

It was not possible to test this explanation by attempting to anneal a labelled probe for the MMTV LTR directly to gel fractionated RNA, because virus-specific RNA transcribed in a normal fashion from proviral DNA was vastly more abundant than transcripts from *int*-1. We attempted to circumvent this problem by using the 'sandwich hybridization' procedure[10,16] to test for RNA molecules containing MMTV U3 sequences covalently linked to *int*-1 sequences. Restriction fragments of phage DNA containing a portion of the *int*-1 domain were fractionated in an agarose gel, transferred to nitrocellulose filters, and annealed with unlabelled RNA from tumour 12 (containing a 3.2 kb transcript) or from tumour 13 (with a conventional 2.6 kb transcript). After removal of unannealed RNA, the filter was incubated with a ^{32}P-labelled *Pst*I fragment containing virtually all of the MMTV LTR. One autoradio-

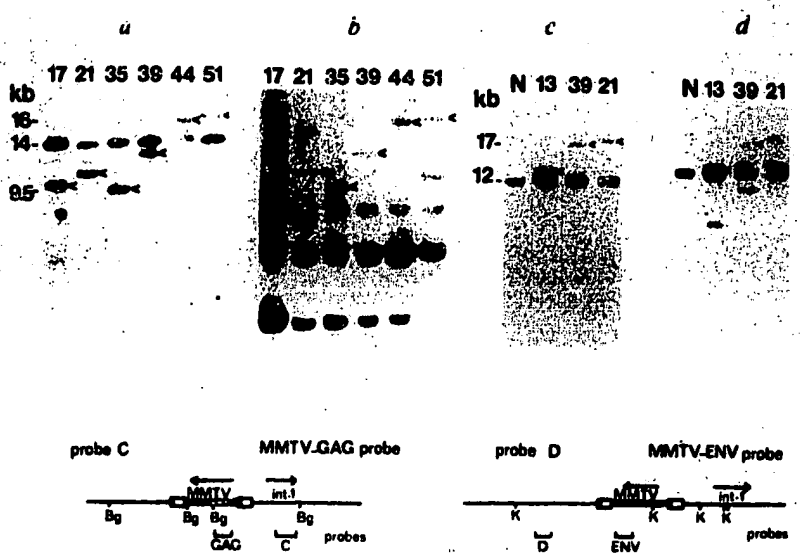

Fig. 1 MMTV proviruses on the 5' side of the *int*-1 transcriptional unit are in the transcriptional orientation opposite to that of *int*-1. DNA samples (10 μg) from mammary tumours (numbers indicated on top) were digested with *Bgl*II (Bg, panels *a* and *b*) or *Kpn*I (K, panels *c* and *d*), electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose[13]. The filters were first incubated with probe C (*a*) or probe D (*c*), washed and exposed to X-ray film. Subsequently, the probes were removed from the filters and the filters were incubated with MMTV-*gag* probe (*b*) or with MMTV-*env* probe (*d*). The origin of the probes is indicated in the diagrams at the bottom of the figure and in Fig. 3; MMTV-*gag* (3.2 kb) is a *Pvu*II–*Bgl*II fragment obtained from a clone of proviral DNA endogenous to GR mice (ref. 56); MMTV-*env* (1.8 kb) is a *Pst*I fragment derived from MMTV (C3H) DNA and cloned into pBR322 (ref. 57). Arrows indicate tumour-specific bands that show rearrangements of the *int*-1 locus detected with probes C and D and bands in the same position detected with the MMTV probes.

graphic signal, at the position of the *int*-1 fragment homologous to probe C, was observed in the experiment conducted with RNA from tumour 12; no reaction was detected with the sample from tumour 13 (Fig. 4*c*). From this experiment we conclude that *int*-1 and MMTV sequences are covalently joined in transcripts from tumour 12; we presume, but have not proved, that the MMTV sequences are located at the 3' end of the 3.2-kb transcript in this tumour.

C nservation of the *int*-1 locus

We have proposed that the *int*-1 domain harbours an oncogene that contributes to mammary tumorigenesis following transcripti nal activation by proviral integration. However, the locus lacks homology to all tested retroviral oncogenes including *sis*, *ski*, *fos*, *fms* and *yes* (data not shown), as well as the 11 examined previously[13], and it does not anneal with the NIH/3T3 cell transforming gene detected in mammary tumour DNA by Lane *et al.* (ref. 17 and our unpublished results with M. A. Lane and G. M. Cooper). Moreover, we have been unable to measure *int*-1 RNA in a variety of tissues from mature mice (liver, spleen r brain), in cultured mouse cells, or in normal mammary glands fr m pregnant or lactating females (data not shown). Since no protein product of *int*-1 has been identified, we sought support f r the idea that the *int*-1 region contains a coding domain by testing for conserved *int*-1 sequences, homologous to probe C, in the DNA of other vertebrates. As illustrated in Fig. 5, *Bam*HI fragments that anneal effectively with probe C in conditions of nly moderately reduced stringency are present in digests of DNA from fish, birds and several mammals, including man. These results are similar to those observed with conserved cellular genes, including the known oncogenes[18]. In addition, as found for the progenitors of some retroviral oncogenes[19], sequences homologous to *int*-1 can be detected in the genome of *Drosophila melanogaster* (lane 1).

Mouse chromosome 15

Specific karyotypic anomalies, some involving putative oncogenes, have recently been found to be associated with a number of different ne plasms[20]. Trisomy of m use chromosome 13, for example, is frequently observed in MMTV-induced mammary tumours[21]. It was therefore of interest to determine the chromosomal location of *int*-1. To assign *int*-1 to a specific m use chromosome, we used probe C to detect restricti n fragments c ntaining *int*-1 in digests of genomic DNA from 30 Chinese hamster–mouse s matic cell hybrid clones segregating

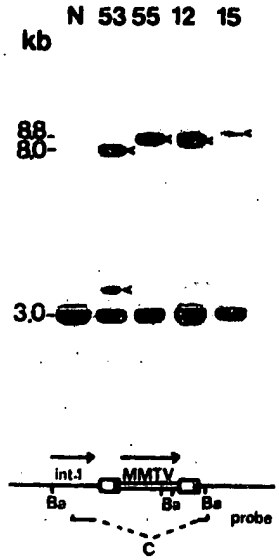

Fig. 2 Proviruses on the 3' side of *int*-1 are in the same transcriptional orientation as *int*-1. Tumour DNA (number indicated on top, N: normal DNA) was digested with *Bam*HI (Ba), transferred to nitrocellulose filters and annealed with probe C. The sizes of the arrowed, novel restriction fragments and additional mapping data (not shown) show that the proviruses were integrated as drawn at the bottom of the figure. This illustration shows the provirus from tumour 53, providing an explanation for the two novel *int*-1 fragments detected with probe C. Proviruses in the other tumours are located further rightward (see Fig. 3).

mouse chromosomes. These hybrids together contain the full complement of murine chromosomes except for chromosomes 11 and Y (ref. 22). The mouse chromosome complement f each hybrid clone was determined by isozyme and/or karyotype analyses as previously described[22]. The single *int*-1-specific *Eco*RI fragment f 8.6 kb present in digests of genomic mouse DNA c uld be easily distinguished from the *int*-1-specific *Eco*RI fragment of 2.8 kb derived from Chinese hamster DNA (not sh wn). An analysis f the segregati n of murine *int*-1 and enzyme markers for each of 16 mouse chrom s mes (all but 3, 5, 11, 13 and Y) revealed 96% c ncordant segregati n of *int*-1 and arylsulphatase A (AS-1), an enzyme marker for mouse chrom s me 15, but discordant segregation f *int*-1 and the

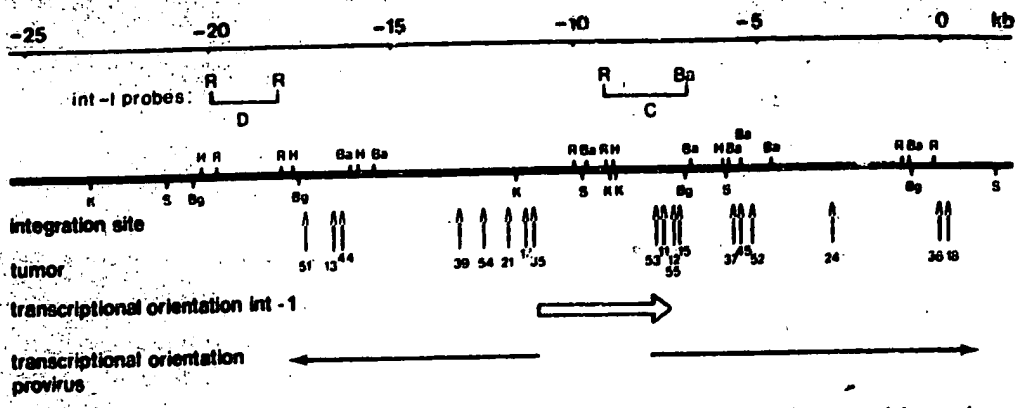

Fig. 3 A map of the proviral integration sites in the *int*-1 locus in C3H mice. Restriction enzyme sites were mapped using C3H and BALB/c chromosomal DNA and DNA from several clones of recombinant bacteriophage containing overlapping inserts from normal BALB/c DNA. Position 0 is defined as the insertion site of the MMTV provirus in tumour 18; this provirus was originally cloned with its flanking DNA to obtain DNA from the *int*-1 locus. Other molecular clones, encompassing positions −22 to +20 on the *int*-1 map, were obtained by sequential screening of a Charon 4A bacteriophage library of normal BALB/c mouse DNA with probes from the *int*-1 region, as described previously[13]. R, *Eco*RI; Ba, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; S, *Sac*I; H, *Hin*dIII. Probes C and D are fragments of single copy DNA positioned as indicated on the map. Vertical arrows above tumour numbers designate sites of integration. The wide horizontal arrow denotes transcriptional orientation of *int*-1 and the approximate extent of the transcribed domain. The narrow horizontal arrows show the direction of transcription of proviruses integrated in the regions demarcated by the arrows.

other enzyme markers (Table 1). When probe C was removed from the DNA blots and the samples reannealed with a probe for the c-*myc* oncogene (data not shown), we observed perfect concordance between *int*-1 and the mouse *Eco*RI DNA fragment derived from the c-*myc* locus previously assigned to mouse chromosome 15 (refs 23, 24). This finding, consistent with the isoenzyme results, provided further evidence for the assignment of *int*-1 to mouse chromosome 15. Karyotype analyses of 12 selected hybrid clones ruled out an association between *int*-1 and chromosomes 3, 5, 11 13 or Y and were also consistent with the assignment of *int*-1 to chromosome 15 (Table 1). The single discordant clone, which was weakly positive for AS-2 but negative for *int*-1, revealed no evidence of a recognizable mouse chromosome 15. Presumably, this hybrid contains a small, non-recognizable fragment of chromosome 15 which includes AS-2 but not *int*-1. Taken together, these data indicate that *int*-1 is located on mouse chromosome 15.

Implications

The results described here support our previous contention that tumour induction by MMTV involves insertion mutations that activate a previously unknown gene called *int*-1. At the same time they promote confidence in the general proposition that oncogenic viruses lacking viral oncogenes may initiate tumour gr wth by insertional mechanisms. This hypothesis fosters an experimental strategy for isolation of oncogenes that is formally analogous to what has been termed 'transposon tagging' in other systems[25]: identification of the mutagenic provirus in tumour DNA should lead directly to the isolation of a linked, mutated cellular oncogene. These ideas now receive support from studies f avian B-cell lymphomas induced by at least two different retroviruses[7,11], erythroleukaemia induced by an avian virus that also causes B-cell lymphoma[26], T-cell lymphoma induced by murine leukaemia virus[27] and mammary tumours induced by MMTV[13,28]. At present only circumstantial evidence implicates the affected cellular genes in the oncogenic process: (1) mutation of the same gene in several tumours of the same type; (2) augmented expression and (3) in some cases homology to progenitors of retroviral oncogenes.

Application f th transposon tagging m thod has led to the discovery of at least tw potential mammary ncog nes: the *int*-1 gene commonly implicated in tumours arising in C3H mice and th *int*-2 d main recently found by Peters *et al.*[28] to be interrupted by proviral insertions in about half of the mammary tum urs arising in the BR6 m use strain. These two targets lack evidence of homology with known retroviral oncogenes r with each other, and they are located on different chromosomes (C. Dickson and R. N., unpublished results). In addition, since substantial numbers of tumours lack insertions in either domain, there may be yet other targets for MMTV insertion mutations.

The case for oncogenic roles for *int*-1 and *int*-2 rests primarily on the argument that MMTV proviral DNA appears capable of integration into many different regions in the mouse genome; henc epeated finding of proviruses at these two loci implies that the tumours are clonal outgrowths of cells[29] with a growth advantage conferred at least in part by the insertion mutations. The case for *int*-1 also rests on the evidence that the locus is transcriptionally active in tumours bearing *int*-1 mutations but not in normal tissues. Although a protein product of *int*-1 has not been identified, a coding function is implied by the conservation of at least part of the transcribed domain during vertebrate evolution (Fig. 5); these findings conform to those obtained with the cellular genes accepted as potential oncogenes by virtue of their homology with retroviral oncogenes.

Our assignment of *int*-1 to mouse chromosome 15 does not offer additional insight into its role as an oncogene: karyotypic abnormalities involving this chromosome have not been reported in mouse mammary tumours, and no endogenous MMTV proviruses have been assigned to chromosome 15 (ref. 30). However, an unrelated oncogene, c-*myc*, also present on mouse chromosome 15, recombines with an immunoglobulin locus on chromosome 12 to produce the translocations found in several plasmacytoma lines[24,31]. Although the distance between *int*-1 and c-*myc* on mouse chromosome 15 is not known, mammary tumours with proviral insertions at *int*-1 do not display abnormal expression of c-*myc* (unpublished data of authors).

The core of the present report concerns the mechanisms by which MMTV proviruses activate expression of *int*-1. In ALV-induced bursal lymphomas, augmented expression of c-*myc* is most commonly achieved by a mechanism referred to as promoter insertion: ALV proviral DNA on the 5′ side of c-*myc* exons in the same transcriptional orientation provides an efficient transcriptional promoter in the form of an LTR[7–10]. However, in the 19 MMTV-induced mammary tumours studied here we have found no instance in which a promoter insertion mechanism activates expression of *int*-1. Instead, MMTV proviruses are positioned either on the 5′ side of the transcriptional template for *int*-1 in the opposite orientation or on the 3′ side in the same orientation. (This rule, however, is not inviolable: in one tumour recently obtained from a BALB/cfC3H mouse, a provirus is present on the 5′ side f the *int*-1 transcripti nal d main in the same orientation (R.N., unpublished.)

Pr vious examples of the tw arrangements observed here, in which an LTR cannot serve as promoter for an activated gene, have been described in ALV-induced bursal lymphomas[10]. In these few instances, the effect on expr ssion of c-*myc* was ascribed t a retroviral 'enhancer' element, akin to similar elements first identified in papovavirus genomes[32–38]. Using

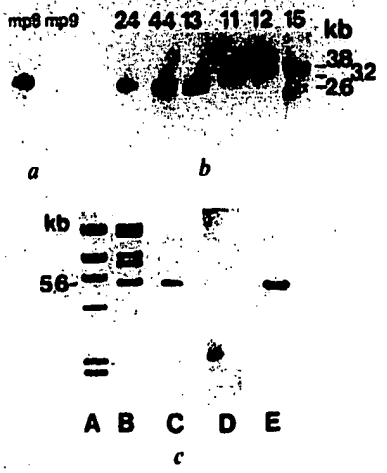

Fig. 4 Analysis of *int*-1 transcription in mammary tumours. *a*, The direction of transcription. 5 μg of polyadenylated RNA from mammary tumour 44 was subjected to electrophoresis in a 1% agarose gel with 2.2 M formaldehyde, transferred to nitrocellulose filters and hybridized with a strand-specific radioactive probe derived by subcloning probe C (a *Bam*HI–*Eco*RI fragment) in the single-stranded bacteriophage derivatives of M13, mp8 and mp9, in both orientations. The autoradiogram shows the hybridization to 2.6-kb RNA with probes made by transcribing the recombinant M13mp8 DNA with reverse transcriptase in the presence of ^{32}P-dCTP with oligomers of calf thymus DNA as random primers. A probe made similarly from the recombinant M13mp9 DNA did not detect a transcript. The direction of transcription of the *Eco*RI–*Bam*HI fragment must proceed from the *Eco*RI site to the *Bam*HI site or from left to right on the *int*-1 map as shown in Fig. 1. *b*, The length of *int*-1 polyadenylated RNA in mammary tumours varies from 2.6 to 3.8 kb. Polyadenylated RNA (5 mg) from mammary tumours with MMTV proviral integrations at the *int*-1 locus was subjected to electrophoresis in 1% agarose gels containing 2.2 M formaldehyde[13] and hybridized with probe C. Tumour numbers are indicated on top (see Fig. 3 for the positions of the proviruses within *int*-1). Tumours 24, 44 and 13 (and also tumours 17, 18, 21, 35, 36, 37, 39, 45) contain a 2.6-kb RNA hybridizing with probe C. Tumour 11 has a 3.2-kb RNA and tumours 12 and 15 have a 3.8-kb RNA species. *c*, Sandwich hybridization demonstrates covalent linkage of *int*-1 sequences to MMTV LTR sequences in tumour 12. DNA from a bacteriophage clone covering the normal *int*-1 region from positions −20 to −3.4 was digested with *Eco*RI and subjected to electrophoresis (lane *B*), next to a marker lane (A) containing wild-type λ DNA digested with *Hin*dIII. DNA was transferred to nitrocellulose filters (several identical filters were prepared from one gel). Direct annealing to radioactive probe C identified a 5.6-kb *Eco*RI fragment (arrow) that contained the *int*-1 transcriptional domain (lane *C*). (This fragment maps from the *Eco*RI site at position −9 in *int*-1 to an *Eco*RI linker at position −3.4.) Other filters were first incubated with unlabelled RNA from tumour 13 (lane *D*) or tumour 12 (lane *E*). These filters were washed and then hybridized to a radioactive probe from the MMTV LTR (a 1.4-kb *Pst*I fragment containing all but 10 bp of the LTR). Only the filter that was annealed first with the RNA from tumour 12 shows hybridization with the *Eco*RI fragment that also hybridized with probe C. Lanes *A* and *B* are photographs of the ethidium-bromide stained gel, the other lanes are autoradiograms.
Methods: 20 μg of poly(A)$^+$ RNA was resuspended in 2 ml of an annealing mix consisting of 50% formamide, 0.5 M NaCl, 20 mM PIPES *p*H 6.8, 5 mM Na_2EDTA, 0.4% SDS, 250 μg ml^{-1} poly(A), 2 × Denhardt's solution, and 200 μg ml^{-1} carrier yeast RNA. This solution was applied to the filter-bound DNA and incubated for 24 h at 41 °C. The filter was washed twice at 55 °C for 15 min with 0.1 × SSC, 0.1% SDS. The filter was blotted dry, annealed to MMTV–LTR probe (2 × 10^6 c.p.m.), washed twice for 1 h at 37 °C with 0.1 × SSC, 0.1% SDS, dried and autoradiographed.

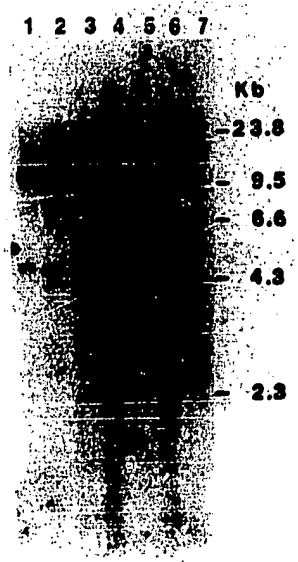

Fig. 5 Sequences homologous to *int*-1 are present in all vertebrates and *Drosophila*. Cellular DNA (10–15 μg) from the indicated sources was digested with *Bam*HI, subjected to electrophoresis in an 0.8% agarose gel, transferred to nitrocellulose filters, and annealed with probe C in 37.5% formamide, 3 × SSC, at 42 °C. After washing in 2 × SSC at 50 °C, the filter was exposed to X-ray film for 6 days. Lane 1, adult *Drosophila melanogaster* (Oregon-S strain); lane 2, fish (*X. helleri*); lane 3, chicken erythrocytes (strain 15 × 7); lane 4, mouse liver (BALB/c strain); lane 5, rat hepatoma cells (H4IIE line, ATCC); lane 6, domestic cat liver; lane 7, human mammary epithelial cells (HBL100 line; ATCC). Numbers at the right (in kb) refer to the positions of *Hin*dIII fragments of λ phage DNA present in the same gel.

DNA transformation techniques, retroviral[39–42] and cellular[43] sequences with enhancer activity have been shown to act in *cis*, over variable distances and independent of polarity, to improve the efficiency of heterologous promoters.

Our evidence that MMTV DNA can activate the expression of a previously silent gene in tumours has several of the characteristics of enhancement: the mechanism of activation operates in a manner independent of orientation, over distances of at least 10 kb, and in *cis* rather than *trans*. The basis for claiming *cis* activation is our observation that insertions between positions −8 and −6 on the *int*-1 map are associated only with transcripts of atypically large size (Fig. 4*b*), despite the persistence of the chromosome bearing a normal *int*-1 allele; we conclude that only the physically rearranged locus has been activated. Other aspects of the situation may also be germane to a consideration of mechanism. (1) The observed effect is conversion of an ostensibly silent gene to one expressed at low levels (less than 10 RNA molecules per cell), rather than to amplify expression of a gene normally transcribed. (2) Enhancer functions are likely to be cell specific[44–49] and perhaps target gene specific, so it is plausible that the MMTV enhancer works preferentially in mammary cells and upon *int*-1. (3) Initiation of MMTV transcription is greatly stimulated by glucocorticoid hormones[50], and the hormonal response displays some of the properties of enhancement[51–53]; hence it is possible that activation of *int*-1 is also affected by physiological doses of hormone. This final point should be amenable to direct test by growing mouse mammary tumour cells with *int*-1 insertions in controlled conditions in culture.

The use of an MMTV enhancer function to activate expression of *int*-1 could explain the uniform orientations of proviruses on each side of the proposed transcriptional unit (see Fig. 3). Enhancers are thought to act only or preferentially upon proximal promoters[54,55]; if the MMTV enhancer is located on the 5′ side of the initiation site for viral RNA, within the 1.2-kb U3 sequence of the LTR, then proviruses would require the orientations observed to avoid interposing an MMTV promoter between the enhancer and its *int*-1 target.

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Correlation between segmental flexibility and effector function of antibodies

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Mouse monoclonal anti-dansyl antibodies with the same antigen-binding sites but different heavy chain constant regions were generated. The extent of segmental flexibility in times of nanoseconds and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgG1 and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.

IMMUNOGLOBULIN G is a flexible Y-shaped molecule. The tw antigen-binding Fab units of IgG are joined to an Fc unit at a hinge that allows the angle between the Fab parts to vary over a broad angular range, as shown by hydrodynamic[1], electron microscopic[2,3], and X-ray crystallographic studies[4–6]. Nanosecond fluorescence polarization measurements have demonstrated that immunoglobulin molecules exhibit segmental flexibility in the nanosecond time range[7–9]. Segmental flexibility is likely to be important in enabling immunoglobulins to bind optimally to multivalent antigens and to carry out certain effector functions. Studies of polyclonal antibody populations have suggested that immunoglobulin classes differ in their degree of segmental flexibility[10–11]. We have explored the relationship between hinge motion and effector function in specially constructed families of homogeneous immunoglobulins. Mouse monoclonal anti-dansyl antibodies with the same antigen-combining sites but different heavy chain constant regi ns (Fig. 1) were generated by selecting somatic variants in hybridoma cell lines. The extent of segmental flexibility in times of nanoseconds (measured by fluorescence spectroscopy) and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least f r IgGl and IgE. Hence, the effector functions f immun globulin isotypes may be controlled in part by the freedom f movement of their Fab arms.

In most antibody-producing hybridoma cell lines, variant cells arise which produce antibody containing a different heavy chain[12]. For example, cells producing IgG2b sometimes arise from a cell line producing IgGl. The frequency of these heavy-chain switch variants is ordinarily low (10^{-5}–10^{-6} per generation). These rare cells can be separated by fluorescence-activated cell sorting on the basis of the appearance of a differ nt heavy chain on their cell surface[13,14]. In these switch variants, the light chain remains the same, whereas V_H becomes joined to a different C_H. Consequently, the antigen-combining site is the same as in the parent, despite the change in the heavy-chain constant region. Many of these switch variants are stable and can be cloned.

We chose to generate a family of homogeneous mouse anti-dansyl (DNS) antibodies for two reasons. First, the fluorescence emission spectrum f the bound dansyl chromophore is very responsive to the polarity of its environment[15], making it a convenient and sensitive indicator of whether the antigen-combining site f the switch variant antibody is in fact the same as that of the parent line. Sec nd, the bound dansyl chromophore has an excited state lifetime suitable f r determining the segmental flexibility of immunoglobulin isotypes by nanosecond fluorescence polarization spectr scopy[16]. Variant hybrid ma cell lines producing different immunoglobulin isotypes were

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WNT-3, Expressed by Motoneurons, Regulates Terminal Arborization of Neurotrophin-3-Responsive Spinal Sensory N urons

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Summary

Sensory axons from dorsal root ganglia neurons are guided to spinal targets by molecules differentially expressed along the dorso-ventral axis of the neural tube. NT-3-responsive muscle afferents project ventrally, cease extending, and branch upon contact with motoneurons (MNs), their synaptic partners. We have identified WNT-3 as a candidate molecule that regulates this process. *Wnt-3* is expressed by MNs of the lateral motor column at the time when MNs form synapses with sensory neurons. WNT-3 increases branching and growth cone size while inhibiting axonal extension in NT-3- but not NGF-responsive axons. Ventral spinal cord secretes factors with axonal remodeling activity for NT-3-responsive neurons. This activity is present at limb levels and is blocked by a WNT antagonist. We propose that WNT-3, expressed by MNs, acts as a retrograde signal that controls terminal arborization of muscle afferents.

Introduction

The formation of neuronal connections requires neurons to project to their appropriate synaptic partners and to make functional synapses. This process is initiated when neurons begin to send axons in search of their targets. Upon reaching the target field, axons branch, cease extending, and their growth cones differentiate into presynaptic terminals. A number of attractive and repulsive signals have been shown to regulate the behavior of axons in transit to their targets (Brose and Tessier-Lavigne, 2000; Cook et al., 1998; Mueller, 1999; O'Leary and Wilkinson, 1999; Perrin et al., 2001). However, little is known about the signals made by postsynaptic cells that regulate the terminal differentiation of presynaptic arbors (reviewed by Tao and Poo, 2001).

The spinal cord has provided a model system to identify the molecules and mechanisms that regulate the formati n of specific neuronal circuits. Secreted molecul s of the netrin, semaphorin, BMP, and Slit famili s guide sensory and commissural axons (Augsburger t al., 1999; Brose and Tessier-Lavigne, 2000; Kennedy et al., 1994; Messersmith et al., 1995; Serafini et al., 1994). Several classes of sensory afferents project from th dorsal root ganglia (DRG) to specific target neurons in the spinal cord (Windle and Baxter, 1936; Mirnics and Koerber, 1995; Ozaki and Snider, 1997). Each class f sensory neurons has a characteristic dorso-ventral projection. Nerve growth factor (NGF)-dependent skin th r-moreceptive/nociceptive sensory neurons make central connections with cells of the dorsal horn (Figure 1B) (Crowley et al., 1994; Ruit et al., 1992; Smeyne et al., 1994). Slit2 regulates axonal extension and collateralization of NGF-dependent DRG sensory neurons (Wang et al., 1999). On the other hand, proprioceptive sensory neurons projecting to the ventral spinal cord (VSC) are of two subtypes: group Ia afferents that carry signals from muscle spindles to motoneurons (MNs; Figure 1B) and group Ib afferents that connect Golgi tendon organs to interneurons in the ventral horn (Light and Perl, 1979; Brown, 1981; Eide and Glover, 1997; Ozaki and Snider, 1997). Thermoreceptive and nociceptive axons, thos that terminate in the dorsal-most layers of the spinal cord, are repelled by members of the collapsin/semaphorin family expressed in the VSC (Fan and Raper, 1995; Giger et al., 1996; Luo et al., 1995; Messersmith et al., 1995; Puschel et al., 1995; Wright et al., 1995). However, muscle afferents that are neurotrophin-3 (NT-3)-dependent (Ernfors et al., 1994; Hory-Lee et al., 1993; Klein et al., 1994; Tessarollo et al., 1994) are not inhibited (Shepherd et al., 1997) and enter the ventral territory. Th stereotypic pattern of projections of muscle afferents suggests that VSC factors, such as F11 (Perrin et al., 2001), regulate their pathfinding. Muscle Ia afferents branch as they enter the ventral horn and, upon contact with MNs, cease extending and form synaptic boutons (Kudo and Yamada, 1987; Chen and Frank, 1999). Explant experiments suggest that VSC provides stop and branching signals for Ia afferents (Sharma and Frank, 1998). However, the molecular identities of ventral horn signals that control the terminal arborization of Ia afferents remain largely unknown.

Recent studies suggest that members of the WNT family of signaling proteins play a role in the formation of neuronal connections. WNT-7A, expressed by cerebellar granule cells, induces growth cone enlargement, axonal spreading, and increases the clustering of synapsin I in mossy fibers (Hall et al., 2000). More importantly, *Wnt-7A* mutant mice exhibit a delay in the morphological maturation and accumulation of synaptic proteins (Hall et al., 2000). Thus, WNT-7A acts as a retrograde signal from postsynaptic neurons to regulate axonal remodeling and expression of synaptic proteins on presynaptic terminals.

Here we have investigated the role of WNT proteins in th formation of th sensory-motor connections in the mouse spinal cord. W show that *Wnt-3* is expressed in MNs f th lat ral motor column (LMC) at a time when sensory axons make contact with th m. WNT-3 inhibits

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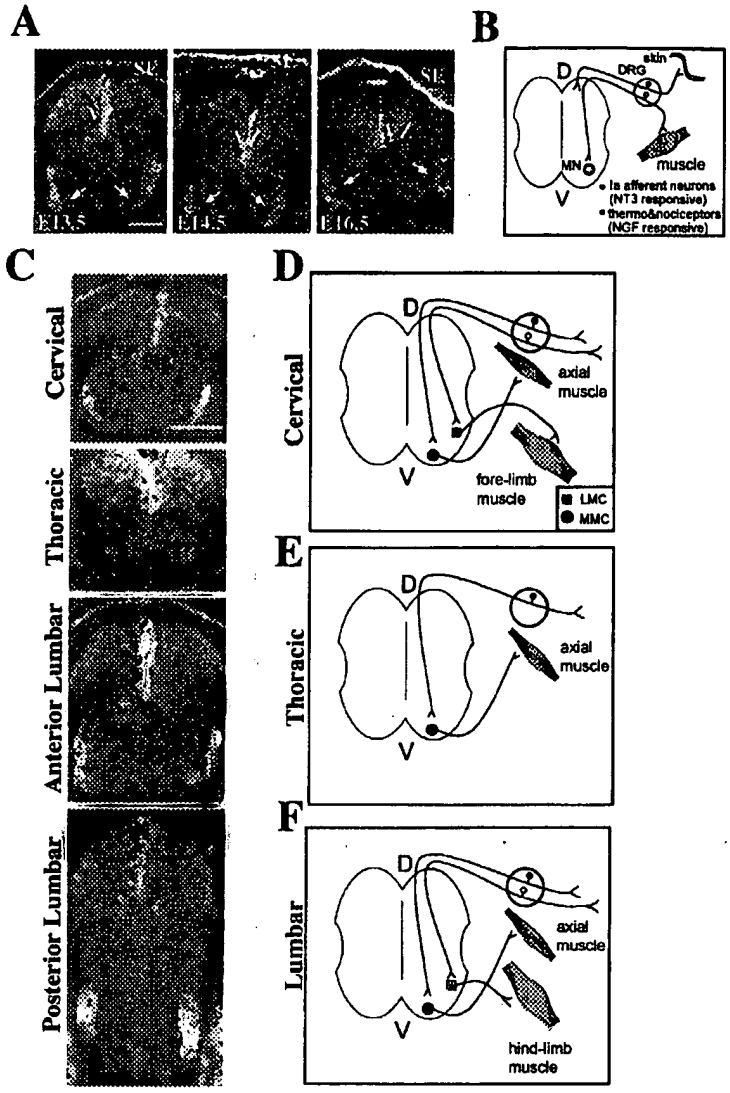

Figure 1. *Wnt-3* Is Expressed in the Lateral Motor Column of the Developing Spinal Cord

(A) In situ hybridization of transverse cervical spinal cord sections shows the expression of *Wnt-3* at E13.5, E14.5, and E16.5. *Wnt-3* expression is detected in the surface ectoderm (SE), ventricular zone (VZ), and in the ventral horn of the spinal cord, where it is restricted to MNs of the LMC. Arrows indicate MNs. Scale bar, 500 μm.

(B) Schematic representation of the sensory neuron projections in the spinal cord. The peak of *Wnt-3* expression in MNs coincides with the time when sensory axons are beginning to form sensory-motor connections. DRG, dorsal root ganglia; MN, motoneurons; D, dorsal; V, ventral.

(C) In situ hybridization of E13.5 mouse spinal cord reveals a restricted expression of *Wnt-3* along the A-P axis. *Wnt-3* expression was found in MNs at cervical and lumbar levels of the ventral spinal cord. No expression of *Wnt-3* was detected in MNs at thoracic levels. Scale bar, 500 μm.

(D–F) Schematic representations of the organization of motor columns and peripheral targets of the MNs at different A-P levels. MMC, medial motor column; LMC, lateral motor column.

axonal extension of DRG neurons. In contrast to other signals that inhibit axonal outgrowth, WNT-3 induces axonal branching and growth cone enlargement rather than growth cone collapse. The effect of WNT-3 is selective for NT-3-dependent sensory neurons, as WNT-3 does not affect NGF-responsive sensory neurons. The specificity of the response matches the expression of *Wnt-3* in MNs at limb levels, the major postsynaptic targets of NT-3-responsive sensory neurons. Interestingly, explants of cervical and lumbar, but not thoracic, VSC are significant sources of axonal remodeling activity that is blocked by the WNT antagonist, secreted Frizzled-related peptide 1 (sFRP1). Thus, our studies identify a WNT-like axonal remodeling activity in the spinal cord that is differentially expressed along the anterior-posterior (A-P) axis. Tak n together, our results suggest that MN-derived WNT-3 acts as a retrograde branching and stop signal for muscle afferents during the formation of sensory-motor circuits in th spinal cord.

Results

Wnt-3 Is Expressed in Motoneurons of the Lateral Motor Column during the Formation of Sensory-Motoneuron Connections

To study a possible role for WNTs in the formation of neuronal connections in the mouse spinal cord, we examined the temporal expression pattern of several *Wnt* genes between embryonic day 12.5 (E12.5) and E17.5. In the developing mouse spinal cord, *Wnt-3*-positive cells are first detected at E12.5 in the ventral horn (Roelink and Nusse, 1991). At E13.5 and E14.5, higher levels of *Wnt-3* expression are detected in the VSC where MNs are found (Figure 1A). Expression of *Wnt-3* declines at E16.5 (Figure 1A) and E17.5 and becomes undetectable from E18.5 (data not shown).

The expr ssion of *Wnt-3* in lateral ventral areas of th spinal cord suggested that *Wnt-3* was restricted to a subset of MNs. Spinal MNs are divided into two large subpopulations, axial and limb MNs (Pfaff and Kintner,

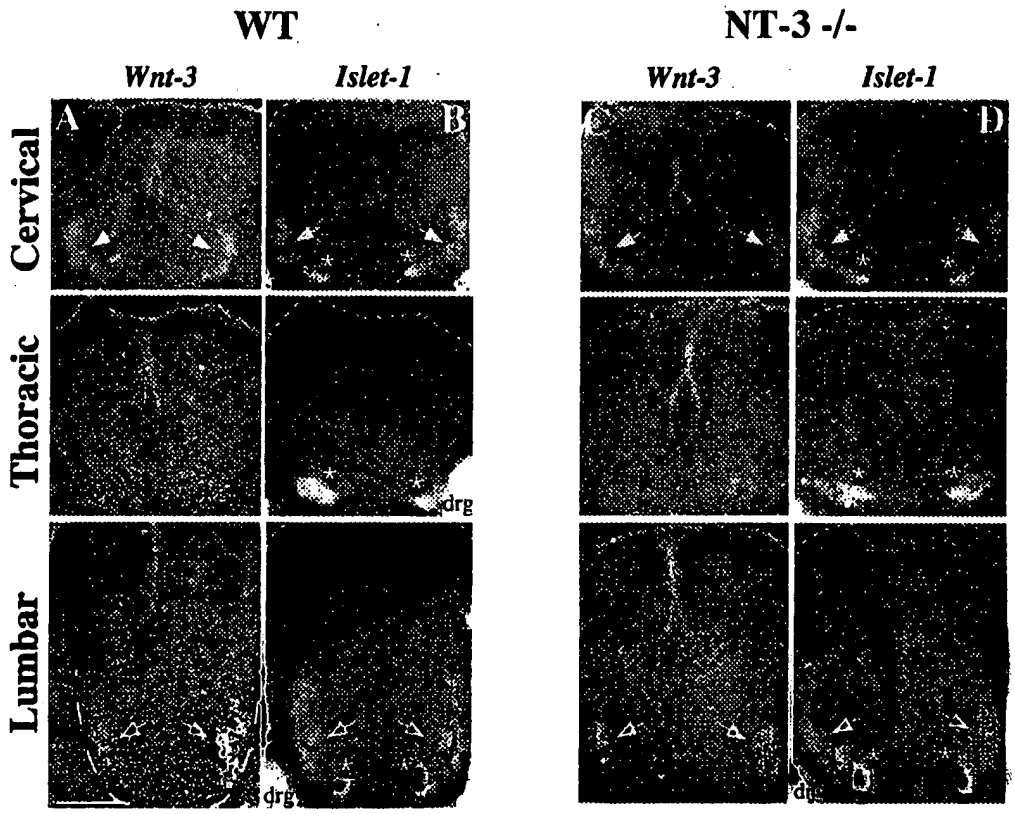

Figure 2. *Wnt-3* and *Islet-1* Colocalize in the Lateral Motor Column of Wild-Type and NT-3 Null Embryos

(A) *Wnt-3* expression along the A-P axis coincides with the expression of *Islet-1* (B) in MNs of the LMC at cervical and lumbar levels of E13.5 wild-type embryos. In contrast, no expression of *Wnt-3* is detected in MNs of the MMC that express *Islet-1* (asterisks). Note the expression of *Wnt-3* in the ectoderm and the ventricular zone and *Islet-1* expression in the DRGs. (C) The pattern and level of expression of *Wnt-3* is not altered in NT-3 null embryos. (D) Expression of *Islet-1* was also normal in these embryos. Dashed line defines the edge of the sections. Arrows, LMC; asterisks, MMC. Scale bar, 500 μm.

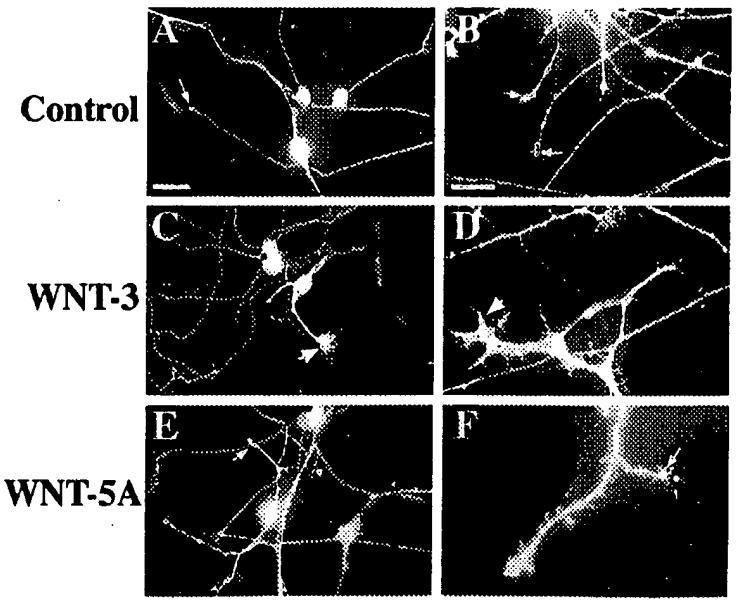

Figure 3. WNT-3 Induces Axonal Remodeling in Sensory Neurons

Embryonic sensory neurons were cultured for 24 hr in the presence of control, WNT-3, or WNT-5A conditioned medium. Cultures were immunostained for GAP-43 to visualize the neuronal morphology.

(A–B) Control sensory neurons have normal morphology with small growth cones.

(C–D) Treatment with WNT-3 induces a significant increase in growth cone size and spreading at the axon terminal.

(E–F) Treatment with WNT-5A does not affect axonal morphology. Large arrows indicate enlarged growth cones. Small arrows indicate small growth cones. Scale bars, 100 μm (A, C, and E); 50 μm (B, D, and F).

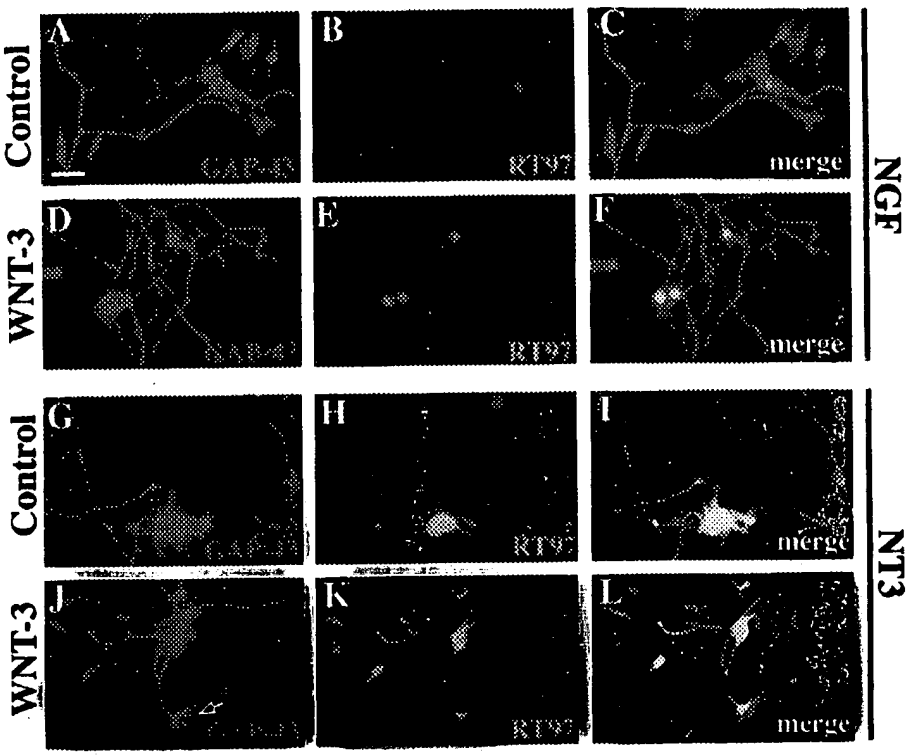

Figure 4. Neurotrophin Selection of Sensory Neurons Is Not Affected by WNT-3

Sensory neurons were grown in the presence of 25 ng/ml NGF (A–F) or NT-3 (G–L) for a total 48 hr. Control (A–C and G–I) or WNT-3-conditioned media (D–F and J–L) were added for the last 16 hr of culture. Staining for GAP-43 (A, D, G, and J) shows the cell morphology. Staining with the RT97 antibody (B, E, H, and K), a marker for NT-3-dependent sensory neurons, labels only the nucleus in NGF-selected cultures (A–F). WNT-3 treatment does not change the number of RT97-stained neurons in either NGF- (D–F) or NT-3-selected cultures (J–L). However, WNT-3 increases growth cone size in NT-3-selected sensory neurons (arrow). Scale bar, 100 μm.

1998; Hughes and Salinas, 1999; Jessell, 2000). Limb MNs form the LMC at cervical and lumbar levels (Figures 1D and 1F) (Landmesser, 1978a, 1978b; Pfaff and Kintner, 1998). To test whether *Wnt-3* expression is restricted to these two populations of MNs, we examined the expression of *Wnt-3* along the A-P axis of the spinal cord. In situ hybridization reveals that *Wnt-3* is expressed mainly in MNs at cervical and lumbar regions at E13.5 and not at thoracic levels (Figure 1). Expression of *Islet-1*, a LIM transcription factor expressed by MNs (Jessell, 2000), shows that *Wnt-3* is detected in the LMC but not in the medial motor column (MMC) (Figures 2A and 2B). This restricted pattern of *Wnt-3* expression along the A-P axis is also observed at E14.5 and E16.5 (data not shown). Furthermore, the onset of *Wnt-3* expression coincides with the arrival of sensory neuron projections to the MN territory and when MNs begin to innervate muscles in the periphery (Altman and Bayer, 1984; Snider et al., 1992; Zhang et al., 1994). Our previous studies have shown that WNT-7A is a retrograde signal capable of remodeling presynaptic axons (Hall et al., 2000). Therefore, a candidate function for WNT-3 made by MNs is to regulate the formation of neuronal connections betw en sensory axons and MNs.

The timing of *Wnt-3* xpression suggests that innervation could regulat *Wnt-3* expression in MNs. NT-3 null mice show sev re loss of NT-3-responsive sensory axons, the presynaptic partners of limb MNs, in the spinal cord (Ernfors et al., 1994; Fariñas et al., 1994). Therefore, we examined the expression of *Wnt-3* in these mice. *Islet-1* expression is normal in both MMC and LMC, suggesting that MN number is not significantly altered in this mutant (Figure 2D). More importantly, in NT-3 null mutant mice, *Wnt-3* expression in spinal MNs is indistinguishable from that in wild-type littermates (Figure 2C). Thus, *Wnt-3* expression in MNs is not dependent upon the arrival of sensory afferents to the VSC.

WNT-3 Induces Growth Cone Remodeling in Sensory Neurons

To test the possible role of WNT-3 as a retrograde signal, we examined whether WNT-3 regulates the behavior of sensory axons. Dissociated DRG neurons isolated from E13.5 mice cultured in the presence of both NGF and NT-3 were exposed to soluble WNT-3 protein. Cells grown in control medium have a normal neuronal morphology (Figures 3A and 3B). In contrast, soluble WNT-3 protein causes an increase in growth cone size in a subset of DRG neurons (Figures 3C and 3D). WNT-5A, which is not expressed in the spinal cord (A. McMahon, personal communication) has no effect on axonal morphology (Figures 3E and 3F). Th se results suggest that WNT-3 has an axonal remodeling activity for a subset of spinal sensory neurons.

WNT-3 Specifically Induces Axonal Remodeling in NT-3- but Not NGF-Responsive Sensory Neurons

Two findings suggest that a distinct population of sensory neurons responds to WNT-3. First, a small proportion of DRG sensory neurons responds to WNT-3 when cultured in the presence of both NGF and NT-3. Second, most of the cells responding to WNT-3 were those with large axon caliber and large cell bodies, characteristics of proprioceptive sensory neurons (Snider and Wright, 1996). Therefore, we used DRG cultures selected with either NT-3 or NGF to test the effect of WNT-3 on proprioceptive (NT-3-dependent) or thermo/nociceptive (NGF-dependent) neurons. NGF-responsive neurons have small cell bodies and fine axons and at E13.5 represent approximately 80% of the total number of DRG sensory neurons, with the remaining 20% being large-diameter NT-3-responsive neurons (reviewed by Snider and Wright, 1996).

As WNT factors have recently been implicated in cell survival (Chen et al., 2001), we first studied whether WNTs affect the number of neurons selected with either NT-3 or NGF. Expression of the neurotrophin receptors, TrkC and TrkA by proprioceptive and thermo/nociceptive sensory neurons, respectively, has been used to identify these two populations of DRG neurons in vivo (Lin et al., 1998; Perrin et al., 2001). The available antibodies against these receptors do not work in dissociated DRG cultures. Therefore, we used the RT97 antibody that recognizes neurofilament proteins and labels large-diameter sensory neurons characteristic of muscle afferents (Lawson et al., 1984; Perry et al., 1991). In NGF-selected cultures, on average, fewer than 10% of the cells were labeled with the RT97 antibody (Figures 4B and 4E). A low level of nonspecific nuclear staining was observed in small diameter cells (Figures 4B and 4E). In control NT-3-selected cultures, over 85% of neurons were RT97 positive (Figure 4H), indicating a substantial enrichment of distinct neuronal populations by neurotrophin selection. The presence of WNT-3 or WNT-5A does not affect the total number of neurons or the number of RT97-positive cells that survive with either NGF or NT-3 compared to control media (Figure 4 and data not shown). These results suggest that WNT-3 and WNT-5A do not affect the survival of a subpopulation of sensory neurons.

We then examined whether WNT-3 selectively affects the morphology of sensory axons. NGF-selected neurons have the same morphology in the presence of control or WNT-3 containing media (Figures 5A and 5C). In contrast, treatment with WNT-3 increases the size of growth cones of NT-3-selected neurons (Figure 5D). Quantification of the growth cone area shows that WNT-3 significantly increases the average size of growth cones (see also Figure 8C). Analysis of the growth cone area distribution shows that WNT-3 induces a shift that results in a decrease of small growth cones, while increasing the percentage of large growth cones ($p < 0.001$; Figure 5F).

Visual inspection of NT-3-selected cultures suggested that WNT-3 also affects axon length in NT-3-selected DRG neurons (Figure 5D). Indeed, WNT-3 induces a 25% reduction in axon length in NT-3-responsive neurons

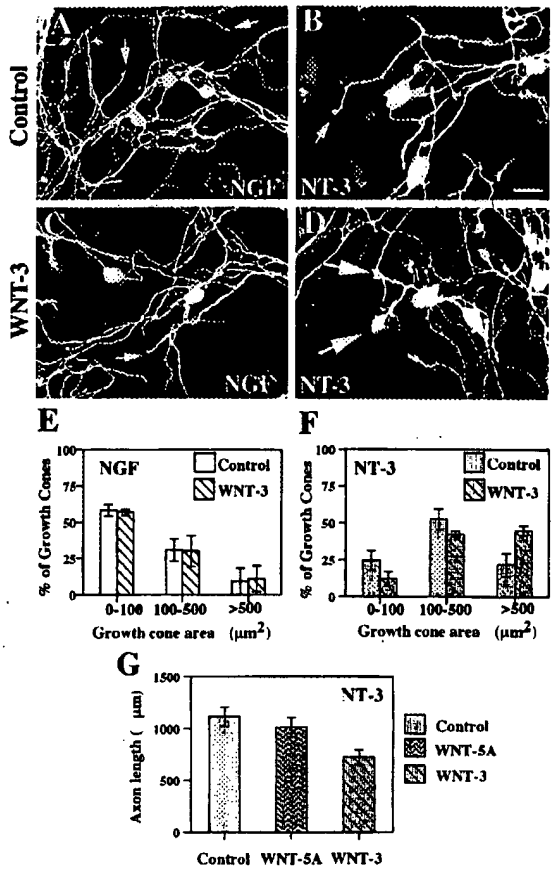

Figure 5. WNT-3 Induces Axonal Remodeling in NT-3 but Not NGF-Responsive Sensory Neurons

Sensory neurons were cultured in the presence of NGF (A, C, and E [unshaded]) or NT-3 (B, D, F, and G) for 48 hr in the presence of control or WNT-3 added for the last 16 hr. Control NGF- (A) or NT-3-selected sensory neurons (B) stained for GAP-43 have small growth cones. (C) WNT-3 does not affect axonal morphology of NGF-selected cultures. (D) WNT-3 treatment induces growth cone enlargement in NT-3-selected sensory neurons. Large and small arrows indicate enlarged and small growth cones respectively. Scale bar, 50 μm. (E) Distribution of growth cone sizes in NGF-selected cultures is unaffected by WNT-3. (F) In NT-3-selected cultures, WNT-3 increases the number of neurons with a growth cone area greater than 500 μm^2, while decreasing the number of growth cones smaller than 100 μm^2 ($p < 0.001$). (G) WNT-3 inhibits axonal extension in NT-3-selected cultures ($p < 0.001$) compared to control or WNT-5A. Values are mean ± SEM, n = 100–150.

($p < 0.001$; Figure 5G). This effect is specific for WNT-3, as WNT-5A does not affect axon length (Figure 5G). In contrast, WNT-3 has no effect on axon length on NGF-responsive neurons (data not shown). These findings demonstrate that WNT-3 induces axonal remodeling in NT-3-responsive sensory neurons.

To further analyze the specificity of WNT-3 effects in NT-3-selected sensory neurons, we examined the role of different WNT factors on axonal remodeling. In addition to WNT-5A (Figures 3E and 3F), we found that WNT-4, WNT-5B, and WNT-7B do not affect the morphology of NT-3-responsive sensory neurons (Figure 6). Although WNT-1 and WNT-6 exhibit a weak axonal re-

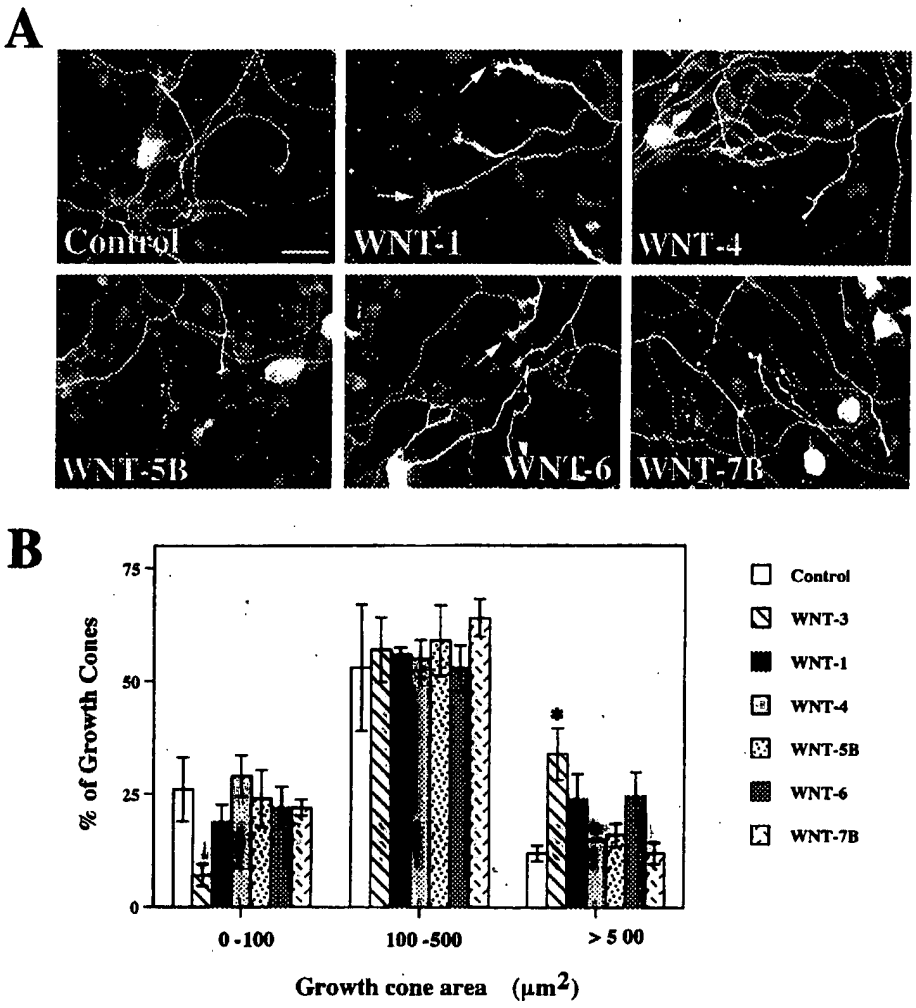

Figure 6. Several WNTs Do Not Affect Axonal Remodeling in NT-3-Responsive Sensory Neurons

Neurons were grown in 25 ng/ml NGF or NT-3 for 48 hr and WNTs added to the culture for the last 16 hr.

(A) NT-3-selected DRG neurons were cultured in the presence of CM containing different WNTs. Arrows indicate slightly enlarged growth cones. Scale bar, 100 μm.

(B) Distribution of the growth cone size in NT-3-selected DRG neurons incubated with various WNT factors. With the exception of WNT-3, none of the WNT factors tested significantly increases the size of growth cones. Asterisk denotes significance for WNT-3 ($p < 0.01$). Values are mean ± SEM, n = 100–150.

modeling activity, these effects were not statistically significant (Figures 6A and 6B). Thus, NT-3-dependent sensory neurons are unresponsive to a number of WNT proteins.

WNT-3 Induces Axonal Branching in Sensory Neurons

In the VSC, sensory axons branch as they reach the motor nuclei (Kudo and Yamada, 1987). Since *Wnt-3* is expressed during this period, we tested whether WNT-3 has a branching activity on DRG neurons. In NT-3-selected cultures, control n urons ar mainly bipolar with few branches (Figure 7A), wher as WNT-3-treated neurons show an increase in the number of branches (Figure 7B). To quantify this effect, we m asured the number of primary and higher order branches per neurite. In NGF-selected cultures, no significant differences were found in the number of primary, secondary, and higher order branches between control and WNT-treated cultures (Figure 7C). In NT-3-selected neurons, WNT-3 increases the number of secondary, tertiary, and higher order branches (Figure 7D), although it does not affect the number of primary branches (data not shown). In contrast, WNT-5A has no effect on axon branching (Figures 7C and 7D). The increased branching suggests that WNT-3 affects the axon shaft, to generate a new area of axon growth. Taken t geth r, our findings indicate that WNT-3 acts as a st p and ax nal branching signal for a s lect population of sensory neur ns, the Ia afferents.

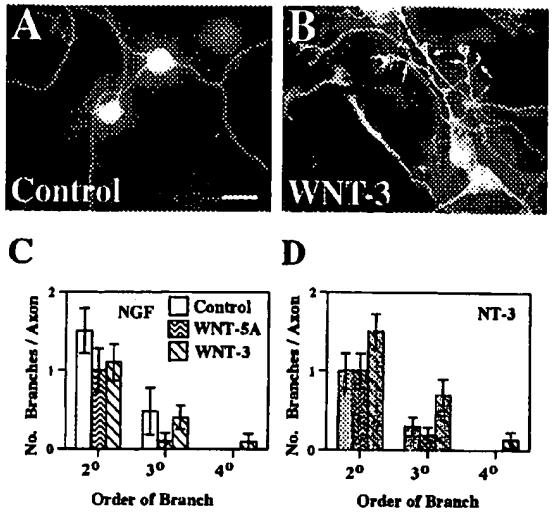

Figure 7. WNT-3 Induces Axonal Branching in NT-3-Responsive Sensory Neurons

(A) NT-3-selected neurons (50 ng/ml for a total 72 hr) treated with control medium are predominantly bipolar with some secondary branching.

(B) WNT-3 induces more secondary branching and the appearance of higher order branches (arrows). Scale bar, 50 μm.

(C and D) Quantification of the number of different order branches per axon in NGF- (C) and NT-3- (D) selected cultures. WNT-5A does not affect significantly the number of branches in NGF- or NT3-selected sensory neurons. WNT-3 does not increase the number of branches in NGF-selected neurons. However, WNT-3 increases the number of secondary order branches in NT-3-selected neurons by 50%, whereas third order branches increase by almost three-fold. Values are mean ± SEM, n = 30–50.

sFRP1 Antagonizes WNT-3 Axon Rem d ling Activity

Du to the lack of purified WNT fact rs, all ur experiments were perf rmed with conditioned media (CM) from xpressing cells. Theref re w analyzed whether the effect observed with WNT-3 CM is due to the direct action of WNT-3 or alternatively due to the presence f another factor induced by the expression of WNT-3. T demonstrate the direct action of WNT-3, we used the secreted WNT antagonist, sFRP1 (Finch et al., 1997; Rattner et al., 1997). In the presence of WNT-3, the average growth cone size was doubled when compared to control cultures (Figures 8A and 8C). However, preincubation of WNT-3 with sFRP1 reverses the effect of WNT-3 by reducing the average growth cone size t control values (Figures 8B and 8C). sFRP1 alone do s not affect axonal morphology when compared to control media (data not shown). We also analyzed the effect f sFRP1 on WNT-3-induced branching activity. WNT-3, when added alone, increases axonal branching (Figures 8A and 8D). We noticed a higher branching activity in these sets of experiments, which correlates with a higher level of WNT-3 in the media (data not shown). Addition of sFRP1 to WNT-3 blocks most of the axonal branching activity of WNT-3 (Figures 8B and 8D), as sFRP1 inhibits the formation of secondary and tertiary branches to almost control levels (Figure 8D). These experiments strongly support the notion that WNT-3 directly mediates axonal remodeling in NT-3-responsive sensory neurons.

WNT-3 Increases Synapsin I Clustering In Sensory Neurons

The changes in the morphology of sensory axons induced by WNT-3 are similar to those observed when

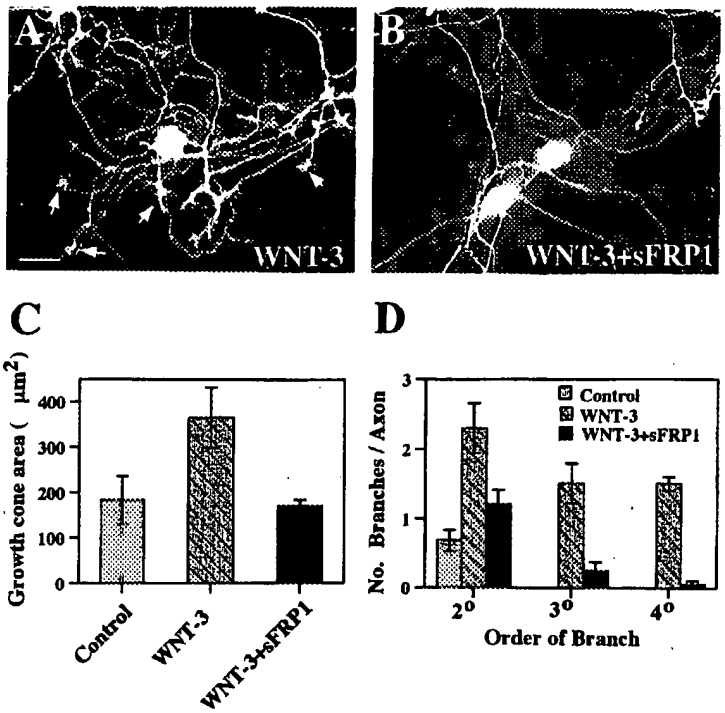

Figure 8. The WNT Antagonist sFRP1 Blocks the Effect of WNT-3 on Axonal Remodeling

WNT-3 CM diluted with control (A) or sFRP1 CM (B) were used to treat NT-3-selected sensory neurons. Arrows indicate enlarged growth cones.

(B) The preincubation of sFRP1 with WNT-3 blocks the effect of WNT-3. Scale bar, 50 μm.

(C) Quantification of the average growth cone area shows that the addition of sFRP1 to WNT-3 reverts growth cone size to that of control values.

(D) The effect of WNT-3 on branching is also blocked by sFRP1, with reduction of secondary and higher order branching to almost control levels. Values are mean ± SEM, n = 50–100.

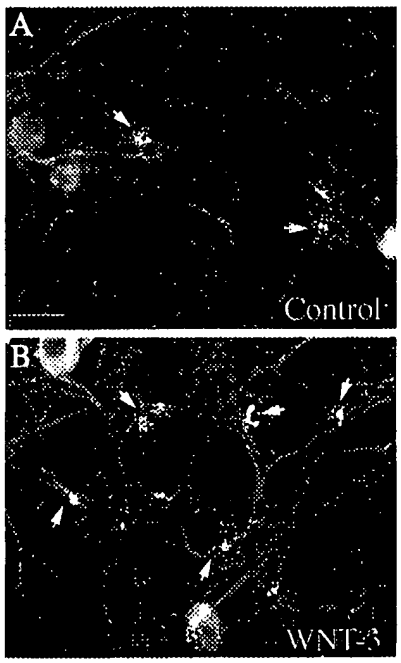

Figure 9. WNT-3 Increases Synapsin I Clustering in Embryonic Sensory Neurons

Sensory neurons were cultured for 6 days in vitro and in the last 16 hr were treated with control or WNT-3 CM.

(A) Control cultures exhibit small synapsin I clusters along the axon and few large clusters in areas where axons cross.

(B) WNT-3 increases the number of large and small synapsin I clusters (arrows) compared to controls. Scale bar, 50 μm.

axons come into contact with their target and begin to form synapses. To begin to address the possible role of WNT-3 in synapse formation, we examined the effect of WNT-3 in the clustering of synapsin I, a presynaptic protein involved in synapse formation and function (Chin et al., 1995; Rosahl et al., 1995). DRG neurons were cultured for 6 days and exposed to control or WNT-3 containing media for the last 16 hr of the culture period. In control sensory neurons, synapsin I is localized in small clusters along the axon and in very few large clusters that formed in areas where several axons cross (Figure 9A). In contrast, neurons cultured in the presence of WNT-3 formed numerous large and small synapsin I clusters (Figure 9B). WNT-3 also increases the overall level of synapsin I along the axon (Figure 9B). The effects of WNT-3 on axonal remodeling and synapsin I clustering suggest a role for WNT-3 in presynaptic differentiation.

Inhibition of GSK-3β by Lithium Mimics the Effect of WNT-3 In Sensory Neurons

The lack of response of NGF-selected sensory neurons to WNT-3 raised the question of how these cells differ from the NT-3-selected population. To address this issue we activated the canonical WNT signaling pathway downstream of th receptor using lithium, a direct inhibit r f GSK-3β (Klein and Melton, 1996). Lithium mimics WNT signaling in early development and during ax nal ext nsion (Hall t al., 2000; Klein and Melt n, 1996; Lucas and Salinas, 1997). Control neurons were cultured in the pr sence f 10 mM sodium chloride. We observed a slight increase in growth cone siz in NGF-selected DRG neurons treated with sodium when compared to control media (compare Figures 5E and 10E). However, sodium chloride treatment does not affect the distribution of growth cones in NT-3-selected cultures (Figures 5F and 10F). Lithium causes growth cone enlargement in NT-3-responsive neurons when compared to sodium ($p < 0.001$; Figures 10B and 10D). Unlike WNT-3, lithium affects NGF-selected neurons ($p < 0.001$; Figures 10A and 10C). In both populations there was a shift in the distribution of growth cone size toward growth cones greater than 500 μm^2 compared to control (Figures 10E and 10F).

Lithium treatment also reduces axon length by 53% in NGF-selected and 48% in NT-3-selected neurons (Figure 10G) and increases axonal branching in both NT-3 and NGF-responsive sensory neurons (Figure 10H). Thus, lithium mimics WNT-3 signaling in NT-3-dependent sensory axons. However, the effect of lithium is not restricted to this specific neuronal population. These results indicate that axonal remodeling is a consequence of GSK-3β inhibition and suggest that NGF-dependent neurons lack functional components upstream of GSK-3β.

Cervical and Lumbar Ventral Spinal Cord Explants Have Growth Cone Remodeling Activity Antagonized by sFRP1

The pattern of *Wnt-3* expression in the LMC and the effect of WNT-3 on NT-3-selected sensory neurons suggest that WNT-3 could act as a retrograde signal from MNs to regulate the formation of motor-sensory neuron connections. To begin to address the role of WNT-3 in vivo, we examined whether VSC at limb levels is a source of WNT-like endogenous activity. Cervical, thoracic, and lumbar VSC explants (Figure 11A) were cultured for 24 hr in serum-free media. CM from these explants were then used to treat NT-3- or NGF-selected sensory neurons. We found that secreted factors from cervical and lumbar levels induce growth cone enlargement in NT-3-selected DRG neurons ($p < 0.001$ for both conditions; Figures 11B and 11D). This effect can be observed by the reduction in the percentage of growth cones with a size less than 100 μm^2 to 15% for cervical and 13% for lumbar compared to 27.5% in control (Figure 11E). Furthermore, growth cones with an area greater than 500 μm^2 increase to 37% and 35% respectively, compared to 20% in control (Figure 11E). The shift in growth cone area distribution is similar to that observed in the presence of WNT-3. In contrast, CM from thoracic levels, where *Wnt-3* expression is undetected, does not have a significant effect on the growth cone size distribution in NT-3-responsive neurons (Figures 11C and 11E). Furthermor , CM from VSC does not affect growth cone size of NGF-responsive neurons (Figure 11F), as b served with WNT-3. These findings suggest that VSC from cervical and lumbar regions secretes factors with a WNT-lik activity.

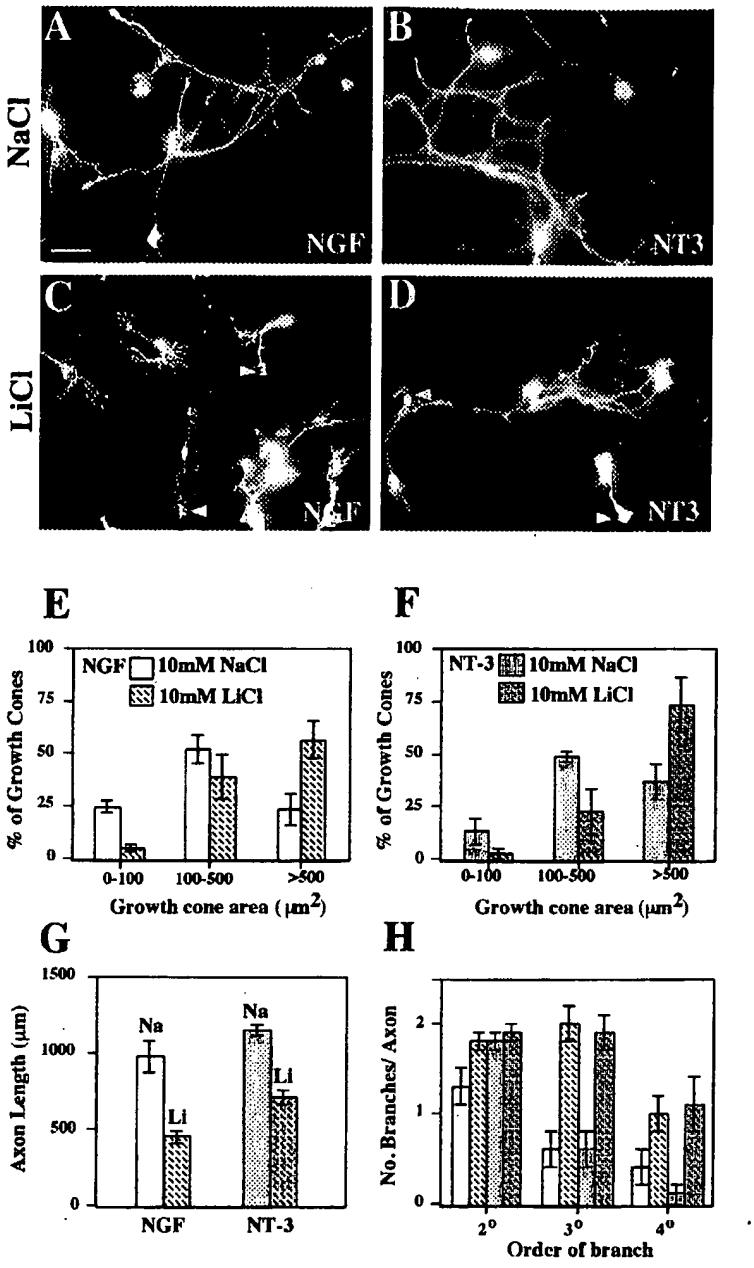

Figure 10. Inhibition of GSK-3β by Lithium Mimics the Effect of WNT-3

Sensory neurons were cultured in the presence of NGF or NT-3 for 48 hr with 10 mM NaCl or 10 mM LiCl added for the last 16 hr. NaCl has little effect on axonal remodeling in either NGF- (A) or NT-3- (B) selected cultures, whereas lithium induces axon shortening, growth cone enlargement (arrowheads), and increased branching in both NGF- (C) and NT-3- (D) selected cultures. Scale bar, 100 μm. (E and F) The distribution of growth cone sizes in NGF- (E) and NT-3- (F) selected cultures is shifted by lithium treatment, with a higher percentage of growth cones greater than 500 μm^2 ($p < 0.001$ for both conditions). (G) Lithium treatment inhibits axonal extension by 53% in NGF- (unshaded) and 48% in NT-3-selected cultures (shaded) ($p < 0.001$). (H) Quantification of axonal branching shows that lithium increases the number of secondary and higher order branches in both NGF- (unshaded) and NT-3-selected cultures (shaded) when compared to control (NaCl). Values are mean ± SEM, n = 100–200.

To investigate if this activity was due to the presence of a WNT protein, we tested whether the WNT antagonist sFRP1 could block the axonal remodeling activity of VSC. We found that sFRP1 blocks the growth cone remodeling activity of cervical and lumbar VSC-secreted factors in NT-3-selected sensory cultures. Addition of sFRP1 to CM from cervical and lumbar explants reverts growth cone size to control values ($p < 0.001$ for both conditions; Figure 11E). Thus, VSC at limb levels has an axonal rem deling activity that can be blocked by sFRP1. Since *Wnt-3* is expressed at c rvical and lumbar l v ls in MNs of the LMC, WNT-3 is likely t mediat at least in part this ndogenous growth cone remodeling activity of the VSC.

Discussion

The formation of functional motor connections in the spinal cord requires matching of specific sensory neurons with their appropriate MN targets. Here we present data that suggest a role for WNT-3 from MNs in regulating the terminal arborization of muscle sensory afferents in the spinal cord. *Wnt-3* is expressed by MNs at cervical and lumbar levels at the tim when sensory n urons come into contact with MNs. WNT-3 inhibits neuronal outgrowth whil increasing growth con siz and axonal branching in NT-3-responsiv sensory neurons, the presynaptic targ ts for MNs. Thus, th behavior induced by WNT-3 res mbl s that observed in sensory axons as

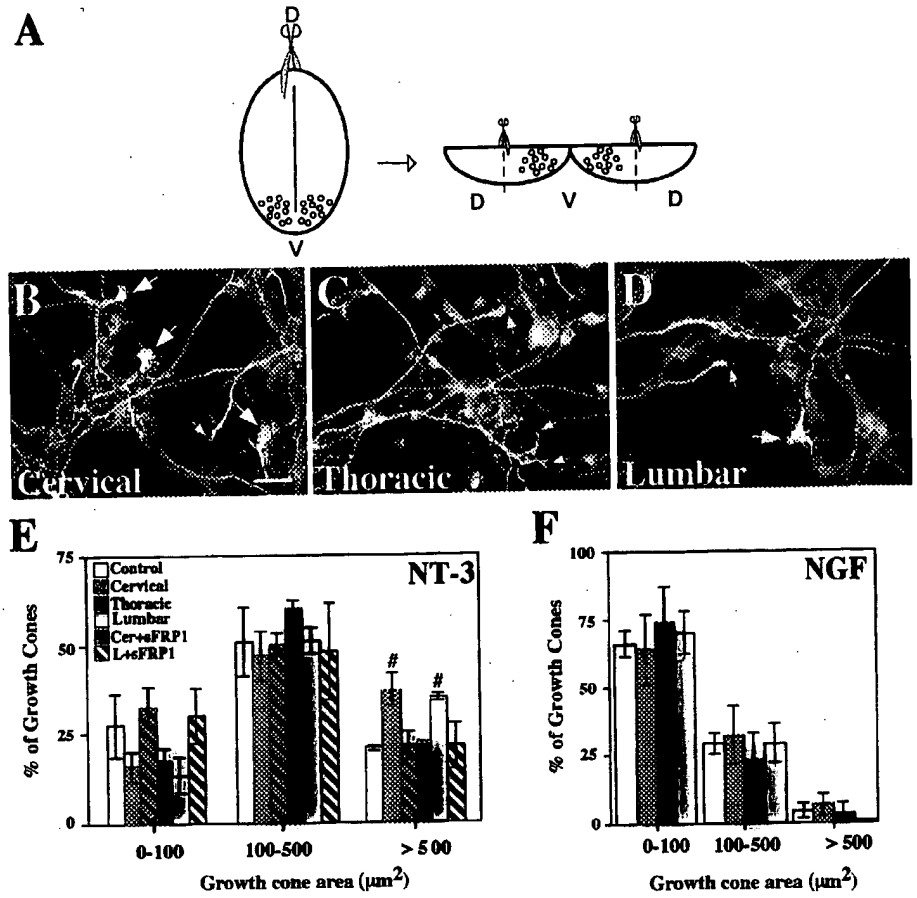

Figure 11. Ventral Spinal Cord at Limb Level Is a Source of Axon Remodeling Activity Blocked by sFRP1

(A) Schematic representation of the isolation of ventral spinal cord explants. Left, the schematic spinal cord viewed in cross-section. Spinal cord was opened from the dorsal side. Small circles denote MNs. Right, the dorsal parts of the resulting plate were dissected out leaving the ventral region containing MNs. D, dorsal; V, ventral.

(B–D) NT3-selected DRG neurons were cultured for the last 20 hr with media conditioned by VSC explants from cervical (B), thoracic (C), and lumbar (D) regions. CM from cervical and lumbar VCS induce growth cone enlargement in NT3-selected neurons (B and D). CM from thoracic VSC explants does not affect axonal morphology of NT3-selected DRG neurons (C). Small arrows indicate small growth cones. Large arrows indicate enlarged growth cones. Scale bar, 100 μm.

(E and F) Distribution of the growth cone size of NT-3- (E) and NGF-selected (F) DRG neurons incubated with CM from E13.5 VSC explants from different A-P levels. Incubation with the CM from cervical and lumbar VSC explants increases the number of growth cones with an area greater than 500 μm^2 in NT-3- selected cultures ($p < 0.001$), as indicated by #. Preincubation with recombinant sFRP1 blocks this effect. The effect of thoracic VSC explants was not significantly different from control. NGF-selected cultures were not affected. Values are mean ± SEM, n = 100–150.

they project to the motor nuclei in the VSC. Importantly, cervical and lumbar VSC explants exhibit a WNT-like axonal remodeling activity that can be blocked by the WNT antagonist, sFRP1. Thus, motoneuronal WNT-3 may act as a selective stop and branching signal for muscle afferents in the developing VSC.

WNT-3 Inhibits Axonal Extension while Increasing Growth Cone Size and Axonal Branching

WNT-3 decr ases axonal length while increasing growth cone area and axonal branching. Th se results sugg st that WNT-3 is bifunctional within the same axon: while inhibiting growth in length of the primary ax n, WNT-3 promot s th growth of branches along the axon shaft. How can these two activities b licited by a uniform concentration of WNT-3? Local differences in the response to WNT-3 may reflect intrinsic differences in cytoskeleton dynamics in different regions of the neurite. Alternatively, WNT-3 could signal through different receptors localized at the growth cone and the axon shaft. We believe that the latter is unlikely, as treatment with lithium, a direct inhibitor of GSK-3β (Klein and Melton, 1996), the kinase that acts downstream of the WNT receptor complex, mimics the effect of WNT-3 at both the growth cone and axon shaft. Lithium, like WNT-3, induces growth cone enlargement and ax n branching and inhibits ax n length. The implications of th se findings are three-fold. First, axonal remodeling induc d by WNT-3 is likely to be mediated through GSK-3β. Second, the ability of lithium in contrast to WNT-3 to induce

axonal r modeling in both NT-3- and NGF-r sponsive sensory neurons suggests that NGF-dependent sensory neurons do not express a functional receptor for WNT-3. Third, the differences in axon growth at the growth con and axon shaft lie downstream of GSK-3β. Differential distribution of GSK-3β targets such as MAPs or other cytoskeleton components along the axon may contribute to the different response to WNT-3. Thus, local changes in composition of the cytoskeleton may explain the different behaviors induced by WNT-3 signaling.

Axon guidance molecules also induce distinct changes in the behavior of the axon shaft and growth cone. Slit proteins increase axonal extension and branching of sensory neurons (Wang et al., 1999). Eph molecules and Semaphorin 3A inhibit axonal extension via growth cone collapse, but promote collateral branching of retinal ganglion cells (Davenport et al., 1999). This contrasts with WNTs and FGF-2 that inhibit axonal extension, yet increase the size of growth cones (Szebenyi et al., 2001; Hall et al., 2000; Lucas and Salinas, 1997). Several mechanisms may contribute to the diversity of responses. One possibility is that signaling molecules affect the actin cytoskeleton and the microtubule organization to different degrees, leading to cessation of axon growth and growth cone collapse in some instances, but inhibiting axonal extension and increasing growth cone size in others. Detailed analyses of the cytoskeleton reorganization and the localization and activation of signaling components in response to different guidance molecules may provide an understanding of the diversity of behavior in developing axons.

WNT-3 and Sensory-Motoneuron Connectivity

Sensory neurons from DRG represent a heterogeneous population of neurons that innervate different targets and respond differentially to signaling molecules (Snider and Wright, 1996). Thermoreceptive and nociceptive sensory neurons project to and synapse with neurons of the dorsal horn and do not extend into the VSC (Mu et al., 1993; Ruit et al., 1992) due to the presence of repulsive signals such as semaphorin III (Messersmith et al., 1995). In contrast, muscle afferents are not repelled by semaphorin III and form extensive branches as they navigate to the motor nuclei (Ozaki and Snider, 1997). Recent studies demonstrate that F11/F3/contactin, a member of the immunoglobulin superfamily of cell adhesion molecules, is required for the proper navigation of proprioceptive sensory axons to their targets (Perrin et al., 2001). Upon encounter with the MNs, muscle afferents cease extending and initiate formation of synapses.

Transplant experiments have suggested that short-range signals from the VSC induce the terminal arborizations of muscle afferents (Sharma and Frank, 1998). We show that E13.5 VSC explants contain a WNT-like axonal remodeling activity for a subset of muscle afferents, those that primarily innervate limb MNs. This activity is restricted to cervical and lumbar levels coinciding with the location of limb MNs in the LMC. In contrast, thoracic VSC explants do not exhibit detectable axonal remodeling activity for NT-3-r sponsive neurons. Spinal c rd from thoracic level contains MNs from the MMC that synapse with axial muscles, but not from the LMC. The xpression of *Wnt-3* specifically in th LMC and its effect nly on NT-3-responsive neurons suggest that WNT-3 c ntributes t the ax nal remodeling activity f the VSC. Although the contribution f factors other that WNT-3 cann t be ruled out, our data strongly suggest that WNT-3 is mediating the activity of VSC. First, the expression of *Wnt-3* along the A-P axis of the spinal cord correlates with the presence of the endogenous axonal remodeling activity of the VSC. Second, both WNT-3 and VSC factors affect NT-3- but not NGF-responsive sensory neurons. Third, both activities are blocked by sFRP1, a secreted WNT antagonist. Thus, our studies support the notion that WNT-3 from MNs can act as a retrograde signal that regulates the terminal arborization of the muscle afferents that innervate the LMC.

The peak of expression of *Wnt-3* coincides with th arrival of NT-3-responsive axons to the VSC, raising the possibility that sensory input may regulate *Wnt-3* expression in MNs. Therefore, we used NT-3 null mice which exhibit a severe loss of NT-3-responsive sensory neurons (Ernfors et al., 1994; Fariñas et al., 1994). Th apparent normal expression pattern of *Islet-1* in the VSC supports previous findings that lack of NT-3 does n t significantly affect MN survival (Snider, 1994). The presence of *Wnt-3* mRNA in the LMC of the NT-3 mutant rules out the possibility that *Wnt-3* expression in MNs depends upon sensory innervation.

NT-3, which itself is expressed by MNs, was proposed to regulate terminal arborization of DRG neurons (Zhang et al., 1994; Lentz et al., 1999). However, NT-3 expressed in muscle is sufficient to rescue proprioceptive neurons in the NT-3 null mice, and these undergo extensive terminal arborization in the ventral horn (Wright et al., 1997). These findings argue against the role of motoneuronal NT-3 in the differentiation of Ia afferents and suggest that another signal from MNs, unaffected by the lack of NT-3, regulates sensory arborization. Therefore, the presence of *Wnt-3* in the NT-3 mutant further supports the role of WNT-3 in the formation of sensory-MN connections.

The question remains, however, what is the function of the restricted expression of *Wnt-3*? The expression of *Wnt-3* in the LMC suggests that WNT-3 regulates sensory neurons that synapse with limb MNs but not those that synapse with axial MNs. A unified model for neuronal matching is now emerging (Jessell, 2000) in which cadherins may facilitate the formation of selective synapses (Yagi and Takeichi, 2000; Price et al., 2002). Interestingly, cadherin expression is regulated by ETS, a family of transcription factors that are implicated in moto-sensory neuron connectivity (Price et al., 2002). As WNT factors regulate cadherin expression (Bradley et al., 1993), our results raise the possibility that WNT-3 is involved in synaptic matching between specific sensory neurons and their synaptic MN targets.

The inhibition of axonal extension and increased growth cone size induced by WNT-3 suggests a role for WNT-3 in presynaptic differentiation in sensory neurons. C nsistent with this idea, we found that WNT-3 increas s th clustering of synapsin I, a presynaptic protein involved in synapse formation and function, in sensory neurons. We have previously sh wn that WNT-7A regulates presynaptic differentiation of pontine m ssy fibers (Hall et al., 2000). Thus, the b havior induced by

WNT-3 signaling is consistent with th r l of WNT-3 in the formation of specific sensory-MN synaps s. As *Wnt-3* null mice are embry nic lethal (Liu et al., 1999), establishing the in vivo function of WNT-3 in neuronal connectivity will require the generation of conditional mutants. Our studies on WNT-3 and WNT-7A suggest that WNTs play a general role in the formation of neuronal connections in the vertebrate CNS. The well-characterized WNT signaling pathway provides a unique opportunity to elucidate the mechanisms that control the terminal differentiation of an actively growing growth cone into a functional presynaptic terminal.

Experimental Procedures

RNA In Situ Hybridization

Frozen or wax-embedded embryos fixed in 4% paraformaldehyde were cut in 10–12 μm sections. Specimens were postfixed, treated with 20 μg/ml proteinase K, washed, refixed with 4% paraformaldehyde, acetylated, and dehydrated (Lucas and Salinas, 1997). [^{35}S]-UTP riboprobes were generated from noncoding sequences of *Wnt-3* (Roelink and Nusse, 1991). Sense probes were used in all experiments and showed no signal. The genotype of NT-3 null embryos was determined by DNA blot analysis (Fariñas et al., 1994).

Production of Soluble WNTs and sFRP1

Soluble WNT-3 and WNT-5A were obtained using QT6 cells transiently transfected with a pCS2+ expression vector containing hemagglutinin (HA)-tagged *Wnt-3*, *Wnt-5A*, or green fluorescent protein (control) cDNAs using the Ca^{2+} phosphate method or from stably transfected Rat1B cells. Transfected cells were cultured 16 hr in the presence of serum-free media. The CM was immediately added to sensory neurons, and these were cultured for a further 16 hr. The levels of WNT protein in the CM were determined by Western blotting using an anti-HA antibody (Roche Diagnostics). The higher levels of WNT-3 in the CM correlated with higher axonal remodeling activity.

QT6 cells were transfected with myc-tagged sFRP1 cDNA expressed in pRK5. WNT-3 CM was incubated for 30 min at room temperature with an equal volume of medium conditioned with sFRP1-expressing cells or mock-transfected cells before addition to neuronal cultures. The presence of sFRP1 in the CM was confirmed by Western blotting using the c-Myc antibody 9E10 (Sigma). The dilution of WNT-3 CM with control medium did not alter axon remodeling and branching activities of WNT-3.

Neuronal Cultures

DRGs were isolated from E13.5 mice according to Kleitman et al. (1991). Neurons were plated at 180 cells/mm^2 (10,000 cells/well) in Lab-Tek chamber slides (Nunc) precoated with poly-D-lysine (100 μg/ml) and laminin (50 μg/ml) and cultured in the presence of both NGF and NT3 (50 ng/ml). For neurotrophin selection we used two different methods: embryonic DRG neurons were cultured either in the presence of 25ng/ml NGF or NT-3 (Promega) for 48 hr or, alternatively, in the presence of 50 ng/ml NGF or NT-3 for 72 hr. Neurons were then exposed to CM from control or WNT-expressing cells for the last 16 hr of the culture period. Similar results were obtained using these two selection methods based on RT97 staining. For neurotrophin selection experiments chamber slides were coated with poly-D-lysine only and cells plated at 10,000 or 30,000 cells/well for NGF and NT-3 treatments, respectively. In lithium experiments neurotrophin selection for 48 hr was used. The culture medium was then supplemented with 10 mM LiCl or 10 mM NaCl for the last 16 hr. Treatment with 10 mM NaCl increases slightly the size of growth cones compared to normal media in NGF-selected cultures. For analyses of synapsin I clustering, DRGs, plated at 13,000 cells/well and cultured for 6 days, were treated with CM from control or WNT-3 expressing cells for the last 16 hr in culture.

Ventral Spinal Cord Explants

Spinal cords from E13.5 mice were dissected according to Figure 11A. DRGs, meninges, and dorsal spinal cord were removed before ventral portions of the spinal cord were divided into three segments corresponding to cervical, thoracic, and lumbar regions (Kandel et al., 1991). The cervical segment corresponded to the same A-P position as the 12 most rostral DRGs, thoracic level to the following 7 DRGs, and lumbar corresponding to the 11 most caudal DRGs. Each segment was then cut into pieces ~0.5 mm in length (explants). Five to seven explants from a single region were cultured for 24 hr in 150 μl of serum-free medium. Ventral spinal cord (VSC) CM was added to neurotrophin-selected (25 ng/ml NT-3 or NGF) embryonic DRG cultures between 24 and 44 hr in vitro prior to fixation. Human recombinant sFRP1 was used at 2.5 μg/ml and preincubated with the corresponding CM (30 min at room temperature) before addition to the cultures. Measurements of the growth cone area were performed blind.

Immunocytochemistry

Sensory neuron cultures were fixed in 4% paraformaldehyde and stored in PBS at 4°C. Cells were permeabilized prior to immunostaining with increasing ethanol concentrations and blocked in PBS containing 5% goat serum, 5% horse serum for 1 hr. Cultures were immunostained overnight using polyclonal anti-GAP-43 and monoclonal anti-neurofilament RT97 antibodies or synapsin I monoclonal antibodies (Serotec) followed by a 1 hr incubation with fluorescent secondary antibodies (Vector Laboratories or Molecular Probes).

Image and Statistical Analysis

Sensory neuron cultures were photographed on 1600 ASA Kodak Ektachrome film, scanned into Adobe Photoshop 4.0, and analyzed using the public domain NIH 1.62 Image program (available at http://rsb.info.nih.gov/nih-image). For the quantification of axon length, an individual axon was measured from the axon hillock to the most distant growth cone along a given route. DRG neurons in control cultures were predominantly bipolar. WNTs did not affect the number of primary axons. The number of branches was quantified by defining the first point of divergence from the main axon as secondary branches. The route of the axon was then followed along one of the secondary branches to the next point of divergence and similarly the number of these tertiary branches was counted. Quaternary and higher order branches were also identified and counted along the entire length of the axon. Statistical analysis was performed on the raw measurements data using Mann-Whitney test. Data from at least three independent experiments were used for statistical analysis.

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The role of *Wnt* genes in vertebrate development

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Over the past decade, many potential candidates for molecules involved in pattern formation in the vertebrate embryo have been identified. Manipulation of the expression of some of these factors has generated fascinating results that have allowed investigators to address their roles in embryogenesis. One such family consists of a group of putative cell signaling molecules related to the proto-oncogene *Wnt*-1. An accumulating body of evidence suggests that the Wnt-family plays a major role in several aspects of vertebrate development.

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Introduction

Cell to cell signaling plays a major role in the foundation and organization of embryonic tissues. Three superfamilies of cell signaling molecules, the transforming growth factor (TGF)-β, fibroblast growth factor (FGF) and *Wnt* gene families, have been implicated in these regulatory interactions. This review will concentrate on the recent advances that have been made toward understanding the role of the *Wnt* gene family in the regulation of vertebrate development.

Properties of the Wnt-family of cell signaling molecules

Wnt-1 (originally referred to as *int*-1) was first identified as a locus activated in response to proviral insertion of mouse mammary tumor virus (MMTV) [1]. Cloning of the integration site identified the *Wnt*-1 gene, which was subsequently shown to encode a glycosylated, secreted protein [2,3]. *Wnt*-1 is the vertebrate counterpart of the *Drosophila wingless (wg)* gene [4], which normally functions in pattern regulation during segmentation as well as during other periods of fly development [5]. In addition to *wg*, two other genes related to *wg* have been identified in *Drosophila* (AP McMahon, unpublished data; R Nusse and A Brown, personal communications), and a large number of sequences have been identified in vertebrates (reviewed in [6]), including genes in mouse, chick, *Xenopus*, zebrafish, newt and human. *Wnt*-family members have been best characterized in the mouse where ten genes have been identified, all of which are expressed at some time during embryogenesis (B Parr, G Vassileva and AP McMahon, unpublished data) [7–10,11••]. The proteins encoded by these genes range in amino-acid identity from 50 to 90% and many features are conserved between molecules, including putative peptide sequences and cysteine residues.

Characterization of Wnt protein has thus far been limited to mouse *Wnt*-1 and its *Drosophila* orthologue *wg*. *In vitro* transformation assays in cultured mammary epithelial cell lines and immunohistochemical analysis of wg protein distribution in *Drosophila* embryos, have revealed that Wnt-1 and wg are secreted [12,13•,14•], and that wg signaling regulates the development of neighboring cells (see [15] for a review). Wnt-1 protein tightly associates with either cell surface or extracellular matrix components [2,3] and this interaction may modulate its activity [14•]. Efforts to purify active Wnt-1 have proven unsuccessful in many laboratories and attempts to generate specific, high-affinity antibodies with mouse Wnt-1 have yielded reagents that do not recognize the proteins *in vivo*. Thus, studies in vertebrates have been restricted to molecular genetic approaches which alter the embryonic expression of *Wnt*-1.

The role of Wnt-1 in the formation of the mouse midbrain and cerebellum

Wnt-1 expression is restricted to the developing central nervous system (CNS) during vertebrate embryogenesis [7,11••,16]. In the presumptive midbrain region of the mouse embryo, mRNA can be detected as early as 8 days *post coitum* (d.*p.c.*) (1 somite) and by 14 somites (approximately 9 d.*p.c.*). *Wnt*-1 is expressed in the dorsal part of the midbrain, in a ring of cells (extending from dorsal to ventral) just rostral to the midbrain–hindbrain junction, and in dorsal aspects of the caudal hindbrain and spinal cord. *Wnt*-1 mRNA can be detected in these cells until at least 14.5 d.*p.c.* [7].

Abbreviations

aFGF—acidic-FGF; **bFGF**—basic-FGF; **CNS**—central nervous system; **d.p.c.**—days *post coitum*; **en**—*engrailed*; **ES**—embryonic stem; **FGF**—fibroblast growth factor; **MMTV**—mouse mammary tumor virus; **sw**—*swaying*; **TGF**—transforming growth factor; **wg**—*wingless*.

In order to determine whether *Wnt*-1 expression is required for development of the CNS, two groups have attempted to create null alleles of *Wnt*-1 using homologous recombination in embryonic stem (ES) cells [17,18]. These studies have provided unambiguous evidence for a role for Wnt-1 in cells of the anterior CNS. Both groups disrupted the *Wnt*-1 coding sequence by inserting the neomycin phosphotransferase gene into the second exon, which is predicted to result in the expression of a truncated, and probably inactive, form of Wnt-1. The studies reported by McMahon and Bradley [17] showed that all embryos homozygous for the targeted allele were missing both midbrain and cerebellar structures, and die within 24 h of birth. Thomas and Capecchi [18] observed the same severe phenotype described by McMahon and Bradley. They, however, also obtained a single homozygous mouse that survived until weaning stage and displayed severe ataxia. Histological examination of the brain revealed that only the anterior portion of the cerebellum was missing and that substantial midbrain tissue was present, a phenotype reminiscent of mice homozygous for a spontaneous mutation at the *swaying* (*sw*) locus, which like *Wnt*-1 maps to chromosome 15 [19]. Further investigation has identified *sw* as a new allele of *Wnt*-1 [20••]. This allele has a frameshift mutation in codon 189 that results in the premature termination of the Wnt-1 protein. As both the targeted alleles and the *sw* allele are expected to encode inactive Wnt-1, why are some of these alleles lethal while others are viable? One obvious explanation is that they may not be true null alleles but may instead exhibit weak activity. Direct testing of these alleles for activity will be necessary in order to completely rule out this possibility. Another explanation for the discordant phenotypes relates to the fact that all three mutations have been maintained on different genetic backgrounds. Therefore, there may be epistatic effects on the expressivity of the phenotype. More extensive genetic analysis of these mutants should provide an answer to this question.

A great deal of emphasis has been focused on the cell signaling mechanisms that underlie the loss of both the midbrain and the cerebellum in *Wnt*-1 mutant embryos in the light of certain aspects of wg signaling during segmental patterning of the *Drosophila* embryo. The wg protein regulates the expression of another segment polarity gene, the transcription factor *engrailed* (*en*), which is expressed in neighboring cells [21,22,23••,24••]. Expression of both genes is necessary for the proper specification of parasegments (see [15] for review). Moreover, during early gastrulation, *wg* and *en* are mutually dependent upon each other's expression for maintenance of their transcription. Recent investigations have analyzed the expression of two murine counterparts of the *Drosophila en* gene (*En*-1 and *En*-2) in normal embryos and embryos homozygous for targeted alleles of *Wnt*-1 [11••]. The expression of mouse *En*-1 is seen throughout the midbrain, coincident with the expression of *Wnt*-1. *En*-2 expression is spatially similar to *Wnt*-1, but it is expressed somewhat later. In addition, *En*-1 and *En*-2 are expressed in the rostral hindbrain, an area where *Wnt*-1 is never expressed. In embryos homozygous for targeted alleles of *Wnt*-1, this entire *En*-1/*En*-2 domain is deleted from early to mid-somite stages [11••]. At present it is not clear whether the complete loss of *En* expressing cells in the brain is directly related to the loss of *Wnt*-1 expression, but the correlation is quite intriguing. In this regard, it will be interesting to examine mouse embryos in which both *En* genes have been inactivated. To date, only mutations in the later expressed *En*-2 gene have been reported; however, only a relatively mild phenotype in the cerebellum results from the loss of this gene product [25••].

The developmental outcome of loss of Wnt-1 activity may be pertinent to studies addressing the role of cell–cell interaction in specifying the fate of midbrain cells. When age-matched grafts from the midbrain of a quail embryo are transplanted into the forebrain of a chick host, adjacent host tissue is instructed to develop along a midbrain pathway [26,27•]. Preceding the induction of histologically identifiable midbrain cells, *En* expression is activated in forebrain cells adjacent to the graft [27•,28•]. These results suggest that a secreted molecule emanating from the midbrain graft may be involved in these inductive interactions. Could the midbrain signal be Wnt-1? At present the answer is unclear. The normal activation of *En* expression in *Wnt*-1 mutant embryos [11••] does, however, suggest that the initial activation of *En* expression in the midbrain does not depend on Wnt-1, but on some other factor or factors. Determination of the exact nature of these inductive signals will require further experimentation.

Wnt factors and axis formation in *Xenopus* embryos

In addition to the role that *Wnt*-1 plays in patterning the CNS, *Wnt* genes have been pushed to the forefront of the constantly evolving field of early *Xenopus* axis specification (for a detailed review see [29]); a field previously dominated by members of the TGF-β and FGF families. Initial studies demonstrated that injection of *Wnt*-1 mRNA into the single-cell embryo results in duplication of anterior regions of the body axis [30]. More recent studies have shown that injection of mRNA for either *Wnt*-1 or X*wnt*-8 [31], another *Wnt*-family member, into a ventral vegetal blastomere at the 8–32-cell stage, leads to a complete axial duplication [32••,33••]. Indeed, *Wnt*-1 and X*wnt*-8 RNA injections are sufficient to rescue the complete dorsal axis in embryos in which normal specification is abolished by UV irradiation [32••,33••]. Thus, it appears that these Wnt-family members are capable of inducing a new dorsal axis, a property previously ascribed to Spemann's organizer, a cluster of cells in the dorsal blastopore lip of the early gastrula. Induction of the organizer itself is thought to be carried out by a group of dorsal vegetal cells, the Nieuwkoop center. Similar to the organizer, grafts of the Nieuwkoop center can restore the dorsal axis in UV-treated embryos [34]. The difference between these two groups of cells reflects their distinct fates within the embryo. Cells from the orga-

nizer region assume dorsal mesodermal fates within the new axis, while cells of the Nieuwkoop center are not found in the rescued dorsal axis, but give rise to endoderm. By coinjecting mRNA for either *Wnt*-1 or X*wnt*-8, together with a lineage tracer, it appears that the Wnt activities assayed are more consistent with the inducing activities of the Nieuwkoop center [33••]. Thus, a Wnt factor normally present in the embryo may be responsible for induction of Spemann's organizer. As neither *Wnt*-1 nor X*wnt*-8 are expressed in the embryo at the appropriate time, or in the correct location, interested parties are presumably in the process of attempting to clone maternal messages that encode Wnt proteins with similar activities. Thus far, no likely candidates have been reported. Alternatively, it is possible that ectopic expression of Wnt proteins activates a receptor-mediated pathway that does not normally respond to Wnt factors in this way, but which is nonetheless coupled to the same intracellular signal transduction pathways that mediate normal organizer induction.

If a Wnt molecule is responsible for specifying cell fates along the axis of the embryo, it will be critical to determine which factors act downstream of the Wnt activity. Many experiments have implicated a role for members of the TGF-β and FGF families of molecules in the formation of mesoderm and axial structures in *Xenopus* embryos (see [35] for a review). The protein activin, a TGF-β-like factor with potent mesoderm-inducing activity, has been shown to induce a secondary axis when RNA encoding the factor is injected into cleavage-stage embryos, although activin appears incapable of completely rescuing UV-treated embryos [32••]. Recently, it has been shown that a maternal activity resembling that of activin is present in *Xenopus* embryos [36]. Furthermore, RNA encoding an activin receptor has been detected in the oocyte and early embryo [37]. However, specific experiments to address the function of this molecule *in vivo* or to determine if activin and Wnt molecules may interact during early *Xenopus* embryogenesis have not yet been reported. The coincidence of expression of different TGF-β-like molecules and Wnt molecules throughout the development of other vertebrates raises the possibility that there is a conserved interaction between these factors.

Both basic (b)-FGF and acidic (a)-FGF, as well as an FGF receptor, are present in cleavage-stage *Xenopus* embryos (for reviews see [29,35]). Definitive evidence that FGF plays a significant role in the formation of mesoderm *in vivo* has recently come from studies in which expression of a dominant negative form of the FGF receptor results in a marked reduction in mesodermal cell types in the posterior trunk region [38••]. Interestingly, there is evidence that the expression of *Wnt* genes can modify the character of mesoderm induced by bFGF, resulting in the formation of dorsal mesodermal tissue and neurectodermal tissues, in contrast to bFGF alone, which induces only ventral mesodermal cell types [39•]. Whether X*wnt*-8 itself is capable of inducing any mesodermal tissues is unclear [32••,39•], but it appears from these studies that Wnt and FGF factors may act synergistically during mesoderm induction and axial specification.

In addition to growth factors whose activity may be modulated in conjunction with Wnt signaling, Wnt may be involved in the regulation of transcription factors that are important for the specification of cell types along the axis. One candidate would be the product of the *goosecoid* gene [40], a putative transcription factor that is also capable of inducing a second body axis [41••]. The *goosecoid* mRNA is restricted to the dorsal blastopore lip, the site of the organizer, and is induced by activin in mesoderm-induction assays [41••]. Similar experiments to determine if Wnt proteins can also activate *goosecoid* expression are no doubt underway. One might predict that Wnt proteins will also have this property as their activity appears to be capable of inducing the organizer itself. It will be interesting to determine whether *goosecoid* and other genes expressed in the organizer region are directly regulated by Wnt proteins.

Future perspectives

While investigations to date have focused principally on *Wnt*-1, future projects will no doubt start to explore the roles of other family members. In the mouse, for instance, different *Wnt* genes are expressed in distinct patterns at different times during gastrulation, neurogenesis and organogenesis (B Parr, G Vassileva and AP McMahon, unpublished data) [7–10,11••]. It will be interesting to determine the roles these genes play in establishing the pattern of the embryo. In addition, we are only beginning to understand the biological effects of these molecules on different cell types. Do these molecules act as growth factors to control cell proliferation in distinct regions or are they inductive signals capable of regulating cell identity or do they produce some combination of these effects? In addition, it is presumed that Wnt proteins act through a cell surface receptor, yet this molecule, or family of molecules, remains to be identified. Issues concerning the biochemical properties of these molecules have been difficult to address, but many groups are now involved in trying to understand more about the Wnt-signaling pathway. Genetic screens in *Drosophila* have already identified several segment polarity genes that may be involved in the wg-signaling pathway. The homologues of these molecules will provide interesting candidates to study in relation to Wnt signaling in vertebrates. In conclusion, a great deal has already been learned about Wnt molecules by compiling information about their function in many different systems, and even more stands to be gained from this approach.

Ackn wledgement

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A homeodomain protein with similarities in the putative DNA-binding region to two *Drosophila* genes, *gooseberry* and *bicoid*, is expressed in the dorsal organizer at gastrulation. Injection of *goosecoid* RNA into ventral blastomeres leads to the formation of a second body axis. This experiment, together with the localization of endogenous *goosecoid* RNA and the altered expression of *goosecoid* in response to ventralizing or dorsalizing agents and mesoderm-inducing factors, is consistent with *goosecoid* playing a central role in specifying organization properties.

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EXPRESSION NOTE

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Ascidian *Wnt-7* gene is expressed exclusively in the tail neural tube of tailbud embryos

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Abstract In vertebrate embryogenesis, many *Wnt* genes are expressed in the neural tube and play important roles in regional specifications. There are many subfamilies of *Wnt*, and each subfamily shows distinct expression patterns in the neural tube. Ascidian larvae have a dorsal hollow neural tube similar to that of vertebrates. To date, the degree of correspondence between regionality of the neural tubes of ascidians and vertebrates remains unclear. To compare cellular differences in neural tubes, *Wnt* genes can be used as molecular probes. We report here that a new member of the ascidian *Wnt* gene family, *HrWnt-7*, was expressed in the tail neural tube at the early tailbud stage. Moreover, in cross-section, *HrWnt-7* was expressed in the dorsal and ventral ependymal cells.

Keywords Ascidian · *Wnt-7* · Neural tube · Early embryogenesis

In the vertebrate neural tube, many *Wnt* genes are expressed in restricted areas and control its patternings (Gavin et al. 1990; Roelink and Nusse 1991; Salinas and Nusse 1992; Parr and McMahon 1994). Among them, mouse *Wnt-7a* and *Wnt-7b* are expressed at the ventrolateral region of spinal cord and the dorsal region of the brain (Parr et al. 1993). *Xenopus* and *Amphioxus Wnt-7b* are also expressed in the neural tube. *Xwnt7B* is expressed in the dorsal neural tube and epidermis at the tailbud stage (Chang and Hemmati-Brivanlou 1998). *AmphiWnt7b* is expressed first in the lateral region of the neural plate, then in most regions of the neural tube at the neurula stage (Schubert et al. 2000). This neural tube-dominant expression suggests the developmental roles of *Wnt-7* in neural tube specification. However, the function of *Wnt-7* in the neural tube remains to be studied.

Ascidians, which are the most basal chordates, have a simpler neural tube that is considered to be a good model of the vertebrate neural tube. The ascidian neural tube consists of four rows of ependymal cells (Satoh 1994). Analyses of the expression of *HNF-3β* suggest that the ventral row of ependymal cells is homologous to the vertebrate floor plate (Corbo et al. 1997; Olsen and Jeffery 1997; Shimauchi et al. 1997). However, more molecular probes should be tested to confirm the suggestion, and *Wnt* homologs can be used to this end. So far, *HrWnt-5* is the only *Wnt* gene isolated from ascidians (Sasakura et al. 1998). Therefore, we tried to isolate a *Wnt-7* homolog from the ascidian, *Halocynthia roretzi*.

To isolate ascidian *Wnt-7* gene, two degenerate oligonucleotide primers were designed, and RT-PCR for *H. roretzi* poly (A)$^+$ RNA from the early tailbud embryos was performed. A PCR fragment with sequence similarity to the *Wnt-7* was obtained. Using this fragment as a probe, an early tailbud cDNA library was screened to obtain a full-length cDNA clone. This cDNA consisted of 4,178 nucleotides and contained a single open reading frame (ORF) that encodes a polypeptide of 436 amino acids. The amino acid sequence of this protein showed strong homology to the Wnt-7 subfamily. The phylogenetic tree also shows that this protein is a member of the Wnt-7 subfamily, supported by a high bootstrap value (Fig. 1). From these findings, we named this gene *HrWnt-7*. Wnt proteins are secreted proteins, and they have a signal peptide sequence that can be recognized by its hydrophobicity, near the N-terminus. However, near the N-terminus of HrWnt-7 protein, there is no recognizable hydrophobic signal peptide sequence. The mRNA has 5′ UTR of 419 bases, and there are ten stop codons in frame, suggesting that the ORF is complete. The second methionine is at the amino acid residue 222, around the middle region of the ORF. These raised the suspicion that this N-terminus is the result of recombination of cDNAs. To confirm that the cDNA of *HrWnt-7* is derived from a single mRNA, we carried out genomic

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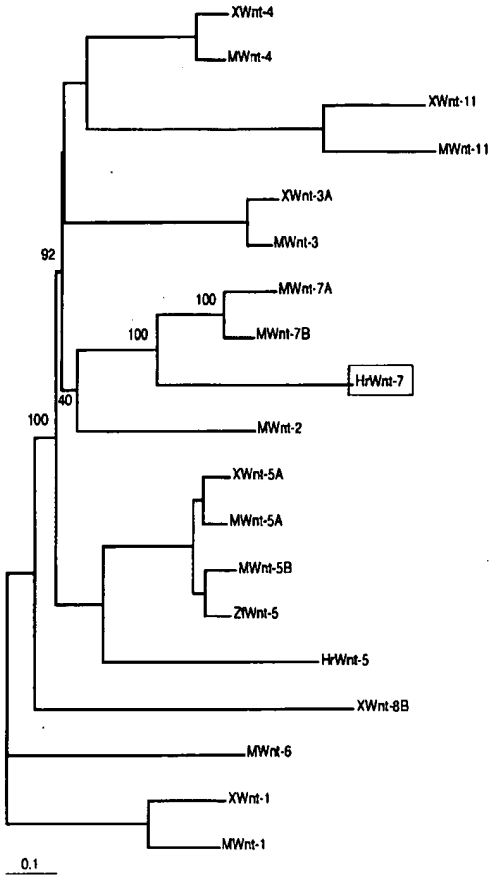

Fig. 1 Molecular phylogenetic tree of Wnt family proteins. This tree was made by the neighbor-joining method (Saitou and Nei 1987). *Bar* indicates an evolutionary distance of 0.1 amino acid substitution per position. The *numbers* at the node represent bootstrap value (%) for the grouping

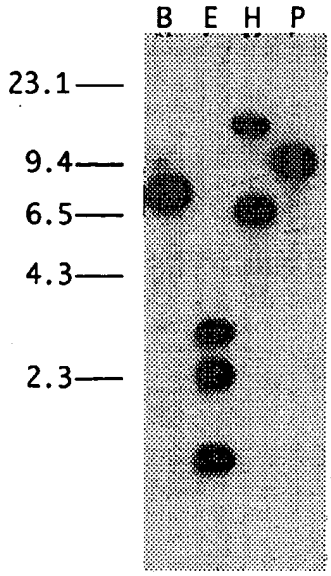

Fig. 2 Genomic Southern blot analysis of *HrWnt-7*. Each lane contains genomic DNA digested separately by *Bam*HI (*lane B*), *Eco*RI (*lane E*) *Hind*III (*lane H*) and *Pst*I (*lane P*). The blots were hybridized with ^{32}P-labeled DNA probes and the membrane was washed under highly stringent conditions. The *numbers* indicate size (in kb) of signals

Southern blot analysis of *H. roretzi* genomic DNA using the *Ssp*I-*Hinc*II fragment of *HrWnt-7* cDNA (which contains the sequence of the N-terminus and the *Wnt*-homology sequence) as a probe. If *HrWnt-7* cDNA is chimeric, more than two bands are always expected in each lane. But a single band was detected in *Bam*HI and *Pst*I lanes (Fig. 2). This finding suggests that this cDNA is derived from a single mRNA, which is a transcript of the *HrWnt-7* gene. In this case, we cannot exclude the possibility that HrWnt-7 protein may not be secreted.

To gain information on the temporal expression of *HrWnt-7*, RT-PCR for the poly (A)$^+$ RNA was carried out. The finding showed that this gene expression started around the 64-cell stage (Fig. 3 A). The amount of transcript increased and peaked around the neurula stage. Then it declined at the tailbud stage and the swimming larval stage.

In addition to the temporal expression, to observe the spatial expression of *HrWnt-7*, whole-mount in situ hybridization was carried out. From the gastrula to the neural plate stage, there were signals in the nuclei of the neural precursors of A-line cells (Fig. 3B, *arrowheads*). The neural precursors of b-line cells seem not to express *HrWnt-7* at this stage. Distinct signals were observed at the neurula stage, at the posterior region of the folding neural plate (Fig. 3C, D). At the early tailbud stage, signals were observed exclusively in the tail neural tube (Fig. 3E, F). From the lateral view, two rows of cells just posterior to the mesenchyme seem to be stained (Fig. 3F, *arrowheads*), suggesting that not all of the ependymal cells were stained. To confirm this, a cross-section of tailbud was generated. The cross-section showed that only the dorsal and ventral ependymal cells expressed *HrWnt-7* (Fig. 3G). Retrospectively, the signals seen in A-line cells in the gastrula are thought to be of descendants of A7.4 (Nishida 1987). This is the first report of a gene that is expressed in both the dorsal and ventral ependymal cells, but not in the lateral cells in the ascidian neural tube.

These findings indicate that the expression of *Wnt-7* in the neural tube during embryogenesis is conserved among chordates. Moreover, both mouse *Wnt-7* and *HrWnt-7* are expressed in the ventral half of the neural tube. However, *Wnt-7*a and *-7b* are not expressed in the floor plate (Parr et al. 1993). Therefore, there is a possibility that the ascidian ventral row of ependymal cells is not fully homologous to the floor plate. Rather, that region may correspond to the ventrolateral region of the vertebrate neural tube. To confirm this possibility, the expression of many more genes should be examined in ascidians. In addition, the functions of *HrWnt-7* in the dorsal and ventral ependymal cells should also be studied.

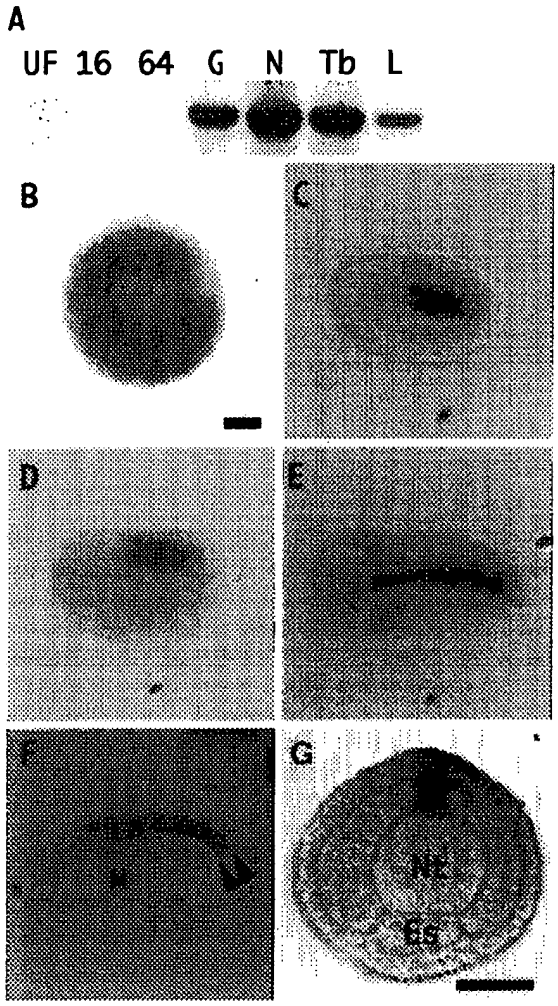

Fig. 3A–G Temporal and spatial expression of *HrWnt-7*. **A** Temporal expression of *HrWnt-7*, as seen by RT-PCR/Southern blot analysis. Each lane represents expression of *HrWnt-7* at the unfertilized egg stage (lane UF), the 16-cell stage (lane 16), the 64-cell stage (lane 64), the gastrula stage (lane G), the neurula stage (lane N), the tailbud embryo stage (lane Tb) and the swimming larval stage (lane L). **B–G** Temporal and spatial expression of *HrWnt-7*, as seen by whole-mount in situ hybridization. **B** A gastrula embryo, vegetal view. Some neural precursors express *HrWnt-7* (*red arrowheads*). **C** A neurula embryo, dorsal view. **D** A neurula embryo, lateral view. **E** An early tailbud embryo, dorsal view. **F** An early tailbud embryo, lateral view. Two rows of cells at the tail neural tube are stained (*arrowheads*). *M* mesenchyme. **G** A cross-section through a tail region of an early tailbud embryo stained for *HrWnt-7*. Signals are seen in the dorsal and ventral ependymal cells. *Nt* Notochord, *Es* endodermal strand. *Bars* 50 μm

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Molecular cloning and characterization of human *WNT3*

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Abstract. Mouse *Wnt-3* is a proto-oncogene, which is activated by mouse mammary tumor virus (MMTV). Human *WNT3* cDNA fragment, previously isolated by another group, corresponds to a partial coding sequence. *WNT3* cDNA, spanning the complete coding sequence, was isolated in this study. *WNT3* encoded 355-amino-acid polypeptide with the N-terminal signal peptide and two N-linked glycosylation sites. WNT3 was most homologous to WNT3A (84.2% total amino-acid identity) among human WNTs. The *WNT3* gene on the human chromosome 17q21 region consisted of five exons. *WNT3* mRNAs were detected in fetal brain, adult brain, and testis by Northern blot analyses. *WNT3* mRNA was relatively highly expressed in A549 cells (lung cancer) and MKN45 cells (gastric cancer) among 37 human cancer cell lines. *WNT3* was significantly up-regulated in a case of primary breast cancer and in a case of primary rectal cancer among various types of human primary cancers. These results strongly suggest that *WNT3* might play a key role in some cases of human breast, rectal, lung, and gastric cancer through activation of the WNT - ß-catenin - TCF signaling pathway, similar to mouse *Wnt-3*.

Introduction

WNT family proteins play key roles in embryogenesis and carcinogenesis (1). WNT signal is transduced through seven-transmembrane recetors with cysteine-rich domain, which are encoded by *Frizzled* (*FZD*) genes (2-9). Genetic alterations of the WNT - ß-catenin - TCF signaling molecules, including *APC*, *AXIN1*, *ß-catenin*, and *TCF-4*, are implicated in human carcinogenesis (10-13). These genetic or epigenetic alterations lead to transcriptional activation of WNT target genes, such as *c-MYC*, *WISP1*, *WISP2*, or *cyclin D1*.

We have previously cloned and characterized *WNT2B/WNT13* (14,15), *WNT3A* (16), *WNT5B* (17), *WNT6* (18,19), *WNT7B* (20), *WNT8A* (21), *WNT10A* (18,19), and *WNT14* (16). Mouse *Wnt-3* is a proto-oncogene, which is activated by proviral insertion of mouse mammary tumor virus (MMTV) (22). Human *WNT3* cDNA fragment, previously isolated by another group, corresponds to a partial coding sequence (23). The complete coding sequence of human *WNT3* gene as well as expression pattern of human *WNT3* mRNA remain to be elucidated.

WNT3 cDNA, spanning the complete coding sequence, was isolated in this study. WNT3 was most homologous to WNT3A (16) (84.2% total amino-acid identity) among members of the human WNT family. The *WNT3* gene consisted of five exons. *WNT3* mRNAs were detected by Northern blot analyses in fetal brain, adult brain, and testis. *WNT3* was relatively highly expressed in human cancer cell lines A549 and MKN45. *WNT3* mRNA was significantly up-regulated in a case of primary breast cancer and in a case of primary rectal cancer among various types of human primary cancers. These results strongly suggest that *WNT3* might play a key role in some cases of human cancer through activation of the WNT - ß-catenin - TCF signaling pathway, similar to mouse *Wnt-3*.

Materials and methods

Blast search. Human genome draft sequences homologous to human *WNT3A* (16) were searched for with the Tblastn program (http://www.ncbi.nlm.nih.gov) as described previously (24,25), in which deduced amino-acid sequence of WNT3A (16) was compared with human genome draft sequences translated in 6 flames.

Poly(A)⁺ RNAs. Poly(A)⁺ RNAs of human fetal brain, lung, liver, kidney, adult stomach, and pancreas (Clontech Laboratories) were purchased. Poly(A)⁺ RNAs of gastric cancer cell lines OKAJIMA, TMK1, MKN7, MKN28, MKN45, MKN74, KATO-III, pancreatic cancer cell lines PANC-1, BxPC-3, AsPC-1, PSN-1, Hs700T, Hs766T, MIA PaCa-2, esophageal cancer cell lines TE1, TE2, TE3, TE4, TE5, TE6, TE7, TE8, TE10, TE11, TE12, TE13, breast cancer cell lines MCF7, T-47D, BT-474, and a colorectal cancer cell line SW480 were extracted with the FastTrack 2.0 Kit (Invitrogen) as described previously (26,27).

One-step cDNA-PCR. Ten ng of poly(A)⁺ RNAs were used as templates for cDNA-PCR with One-step RT-PCR kit (Qiagen) as described previously (28,29). Cycle profiles of cDNA-PCR were as follows; reverse-transcription reaction at 50°C for 30 min, activation of HotStarTaq DNA polymerase

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Key words: WNT, Frizzled, ß-catenin, gastric cancer, pancreatic cancer

Table I. Oligonucleotide primers.

Primer	Orientation	Nucleotide sequence	Nucleotide position
PW3-01	Sense	CGGCGCCTCTTCTAATGGAGC	1-21 of *WNT3*
PW3-02	Anti-sense	GAGAGCCTCCCCGTCCACAG	1259-1240 of *WNT3*
PW3-03	Sense	CTGCCAGGAGTGTATTCGCATC	1037-1058 of *WNT3*
BACT2	Anti-sense	GCGGATGTCCACGTCACACT	943-924 of ß-*actin*
BACT3	Sense	CCACTGGCATCGTGATGGAC	516-535 of *ß-actin*

at 95°C for 15 min, and 17 to 28 cycles of amplification (94°C for 0.5 min, 60°C for 0.5 min, 72°C for 2 min). cDNA-PCR products were purified with the QIAEX Kit (Qiagen), and were ligated into TA cloning vector pCR2.1 (Invitrogen) for subsequent nucleotide sequence analyses with ABI310 Sequencer (PE Applied Biosystems). Nucleotide sequences of PCR primers are listed in Table I.

Northern blot analysis. After pre-hybridization for 1 h at 68°C in QuikHyb solution (Stratagene), MTN Northern blot filters and an RNA master blot filter (Clontech Laboratories) were hybridized with [α-^{32}P]dCTP-labeled W032 probe for 1 h at 68°C in QuikHyb solution as described previously (30,31). After washing, filters were exposed to the Imaging Plate (Fuji) for image analyses with the Storm system (Molecular Dynamics).

Matched tumor/normal expression array analysis. After pre-hybridization for 30 min at 65°C in ExpressHyb solution (Clontech Laboratories), a matched tumor/normal expression array filter (Clontech Laboratories) was hybridized with [α-^{32}P]dCTP-labeled W032 probe at 65°C overnight. After washing, filters were exposed to the Imaging Plate (Fuji) for image analyses.

Results

Isolation of WNT3 cDNAs. Putative exons 2-4 of the human *WNT3* gene were identified on human genome draft sequence AC004098.1 by using the amino-acid sequence of WNT3A (16) as a query sequence for the Tblastn program. Putative exon 1 of the human *WNT3* gene was identified on human genome draft sequences AC015855.5 and AC019319.9 by using the amino-acid sequence of mouse Wnt-3 (22) as a query sequence. Human *WNT3* ESTs (accession nos. AI638412 and AA634318), corresponding to part of ORF and 3'-UTR, were identified by using the nucleotide sequence of putative exon 4 as a query sequence for the Blastn program. W031 cDNA of 1259-bp in size was isolated by cDNA-PCR from a mixture of poly(A)$^+$ RNAs of human fetal brain, lung, liver, and kidney. The W031 cDNA was found to consist of 14-bp 5'UTR, 1068-bp ORF, and 177-bp 3'-UTR.

Amino-acid sequence of WNT3. WNT3 encoded 355-amino-acid polypeptide. The N-terminal hydrophobic signal peptide was identified in WNT3 by using Kyte and Doolittle hydrophobicity analysis (Fig. 1B). Two N-linked glycosylation sites (Asn90 and Asn301) and consensus amino-acid residues conserved among members of the WNT family were identified in WNT3 (Fig. 1C). Among human WNTs, WNT3 was most homologous to WNT3A (84.2% total-amino-acid identity).

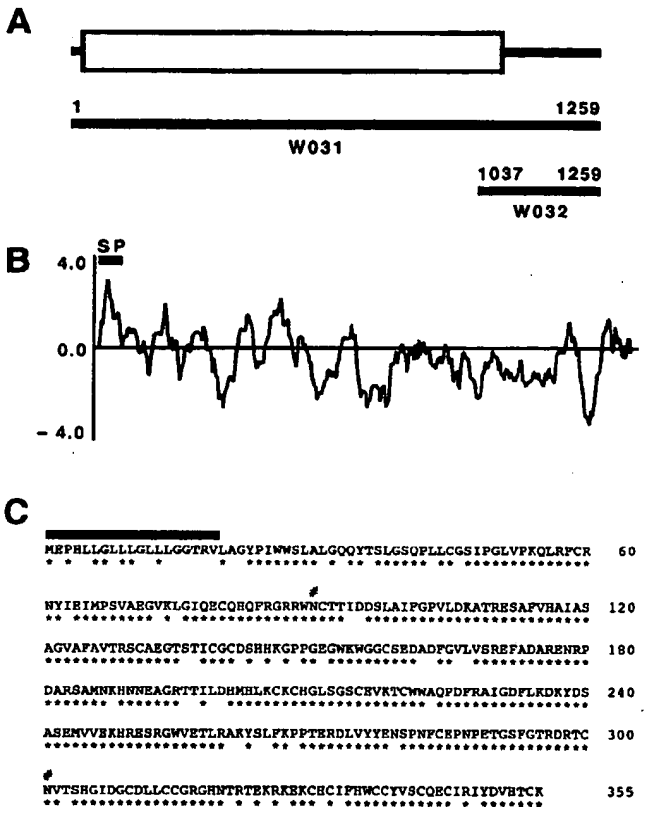

Figure 1. Structure of *WNT3* cDNA and WNT3 polypeptide. (A), Schematic presentation of *WNT3* cDNAs. ORF is depicted as an open box, and UTRs as bold bars. W031 corresponds to nucleotide position 1-1259 of the *WNT3* cDNA, and W032 to nucleotide position 1037-1259. Nucleotide sequence of WNT3 cDNA will appear in the DDBJ/ENBL/GenBank data bases as accession no. AB067628. (B), Kyte and Doolittle hydrophobicity analysis. The N-terminal hydrophobic region (SP) is shown by bold over-line. (C), Amino-acid sequence of WNT3. Amino-acids are numbered on the right. N-terminal signal peptide (bold over-line) and two N-linked glycosylation sites (sharp) are shown above the alignment. Amino-acid residues conserved between WNT3 and WNT3A (asterisk) are shown below the alignment. Total-amino-acid identity WNT3 and WNT3A is 84.2%.

Table II. Structural comparison between *WNT3* and *WNT3A* genes.

Exon No.			Nucleotide and amino-acid sequences around exon-intron boundaries	
			M E P H P I W W	
Exon 1	*WNT3*	5'-UTR	ATGGAGCCCCAC----------CCCAATTTGGTG	gtaaga
	WNT3A	5'-UTR	ATGGCCCCACTC----------CCCGATCTGGTG	gtgagt
			M A P L P I W W	
			S L A L V L D K	
Exon 2	*WNT3*	ctgcag	GTCCCTGGCCCT----------TCCTCGACAAAG	gtactg
	WNT3A	ctgcag	GTCGCTGGCTGT----------TGCTGGACAAAG	gtatgg
			S L A V V L D K	
			A T R E A G R T	
Exon 3	*WNT3*	ccgcag	CCACCCGCGAGT----------GCGGGCCGCACG	gtgagc
	WNT3A	ctacag	CTACCAGGGAGT----------GCTGGGCGCCAG	gtaggt
			A T R E A G R Q	
			T I L D T C K *	
Exon 4	*WNT3*	ccccag	ACTATCCTGGAC------ACCTGCAAGTAGGGCACCAG	gtaggg
	WNT3A	ccgcag	GCCATCGCCAGC------ACCTGCAAGTAGGCACCGGC	3'-UTR
			A I A S T C K *	
Exon 5	*WNT3*	cccaag	GGCGCTGGGAAG----------	3'-UTR

Nucleotide sequence of exon and intron are indicated by large caps and small caps, respectively.

Structure of the WNT3 gene. Exon-intron structure of the *WNT3* gene was determined by comparing the nucleotide sequence of the *WNT3* cDNA with those of human genome draft sequences AC015855.5, AC019319.9, and AC004098.1. The *WNT3* gene was found to consist of five exons (Table II).

Expression of WNT3 in normal human tissues. The W032 probe, corresponding to nucleotide position 1037-1259 of *WNT3* cDNA, hybridized to 1.6-, 3.4-, and 4.6-kb *WNT3* mRNAs. The 1.6-kb *WNT3* mRNA was detected in testis, while 3.4- and 4.6-kb *WNT3* mRNAs were faintly detected in fetal and adult brain (Fig. 2).

Alternative polyadenylation of WNT3 mRNAs. WNT3 ESTs AI638412 and AA634318, polyadenylated at the position 124 bp downstream of W031 cDNA, correspond to the 1.6-kb *WNT3* mRNA. *WNT3* ESTs AW302250 and AI761902, polyadenylated at the position 1951 bp downstream of W031 cDNA, correspond to the 3.4-kb *WNT3* mRNA. *WNT3* EST corresponding to the 4.6-kb *WNT3* mRNA was not identified by the Blastn program probably due to the existence of *Alu* repetitive sequence around the putative polyadenylation site, about 3100 bp downstream of W031 cDNA. These results suggest that at least 1.6- and 3.4-kb *WNT3* mRNAs might be generated due to alternative polyadenylation.

Expression of WNT3 in 37 human cancer cell lines. WNT3 mRNAs of 3.4- and 4.6-kb in size were relatively highly expressed in A549 (lung cancer), and were weakly expressed in HeLa S3 (cervical cancer), K-562 (chronic myelogenous leukemia), MOLT-4 (lymphoblastic leukemia), and SW480 (colorectal cancer) (Fig. 2C).

Expression of the *WNT3* mRNA in gastric, pancreatic, esophageal, and breast cancer cell lines were next investigated by using cDNA-PCR with PW3-03 and PW3-02 primers, which detected *WNT3* cDNA of 223-bp in length. *β-actin* cDNA was almost equally detected in each sample. Amount of *WNT3* cDNA was significantly higher in MKN45 cells (gastric cancer) than in fetal brain or SW480 cells (Fig. 3).

Up-regulation of WNT3 in human primary cancers. Expression of *WNT3* mRNA among 9 cases of breast cancer, 8 cases of gastric cancer, 3 cases of lung cancer, 11 cases of colon cancer, 7 cases of rectal cancer, 3 cases of prostate cancer, 14 kidney tumors, 7 uterus tumors and 3 cases of ovarian cancer was next investigated by using matched tumor/normal expression array filter (Clontech Laboratories). Relatively strong hybridization signal was detected on A549 cells with the W032 probe (data not shown), which was consistent with the result obtained by Northern blot analysis (Fig. 2C). *WNT3* was significantly up-regulated in a case of primary breast cancer and in a case of primary rectal cancer.

Discussion

Human *WNT3* gene encoded 355-amino-acid polypeptide with the N-terminal hydrophobic signal peptide and two N-linked glycosylation sites (Fig. 1C). Comparison between human

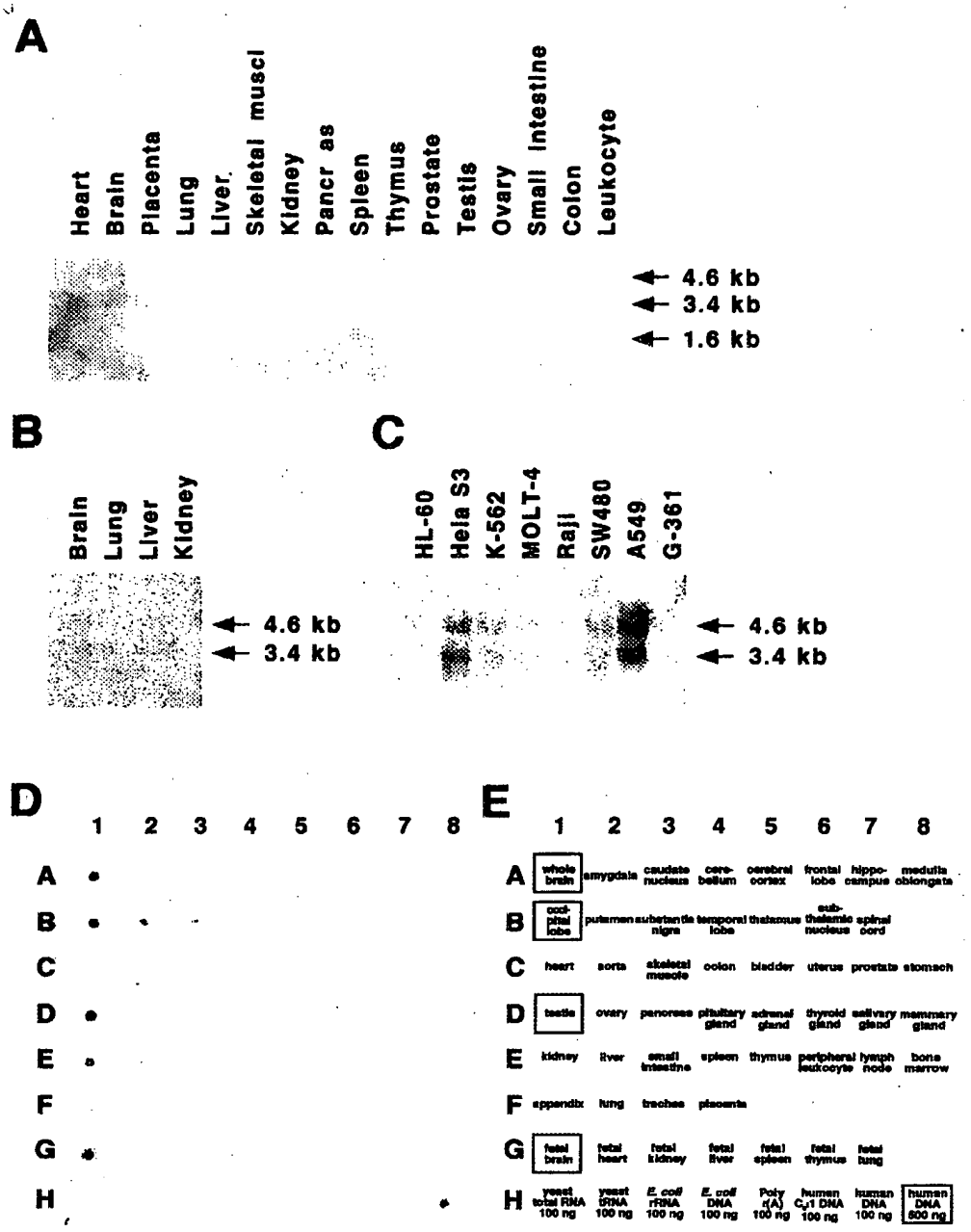

Figure 2. Northern blot analyses and RNA master blot analysis on *WNT3* mRNA. (A), Adult tissues. (B), Fetal tissues. (C), Cancer cell lines. MTN Northern blot filters were hybridized with [α-^{32}P]dCTP-labeled W032 probe. *WNT3* mRNA of 1.6-kb in size was weakly expressed in testis, and *WNT3* mRNAs of 3.4- and 4.6-kb in size were faintly expressed in fetal and adult brain. Among human cancer cell lines, *WNT3* mRNAs of 3.4- and 4.6-kb in size were relatively highly expressed in A549 (lung cancer), and weakly expressed in HeLa S3 (cervical cancer), K-562 (chronic myelogenous leukemia), MOLT-4 (lymphoblastic leukemia), and SW480 (colorectal cancer). (D), RNA master blot analysis. (E), Diagram of RNA master blot filter. RNA master blot filter was hybridized with [α-^{32}P]dCTP-labeled W032 probe. *WNT3* mRNA was detected in fetal brain, adult brain, and testis.

WNT3 cDNA (this study) and human *WNT3* cDNA fragment (23) revealed that *WNT3* cDNA fragment (23) was truncated at codon 333. These results clearly indicate that this is the first report on molecular cloning of human *WNT3* cDNA, spanning the complete coding sequence.

Among human WNTs, WNT3 was most homologous to WNT3A (84.2% total-amino-acid identity). *WNT3* and *WNT14B* genes were clustered in the human chromosome 17q21 region with an interval of about 33 kb, while *WNT3A* and *WNT14* genes were clustered in the human chromosome 1q42 region with an interval of about 58-kb in size (16). *WNT3-WNT14B* gene cluster and *WNT3A-WNT14* gene cluster might be generated due to duplication of an ancestral gene cluster, just like *WNT10A-WNT6* gene cluster and *WNT10B-WNT1* gene cluster (18).

The *WNT3* gene was found to consist of five exons (Table II), while the *WNT3A* gene consists of four exons (16). Exon-intron structure in ORF was well conserved

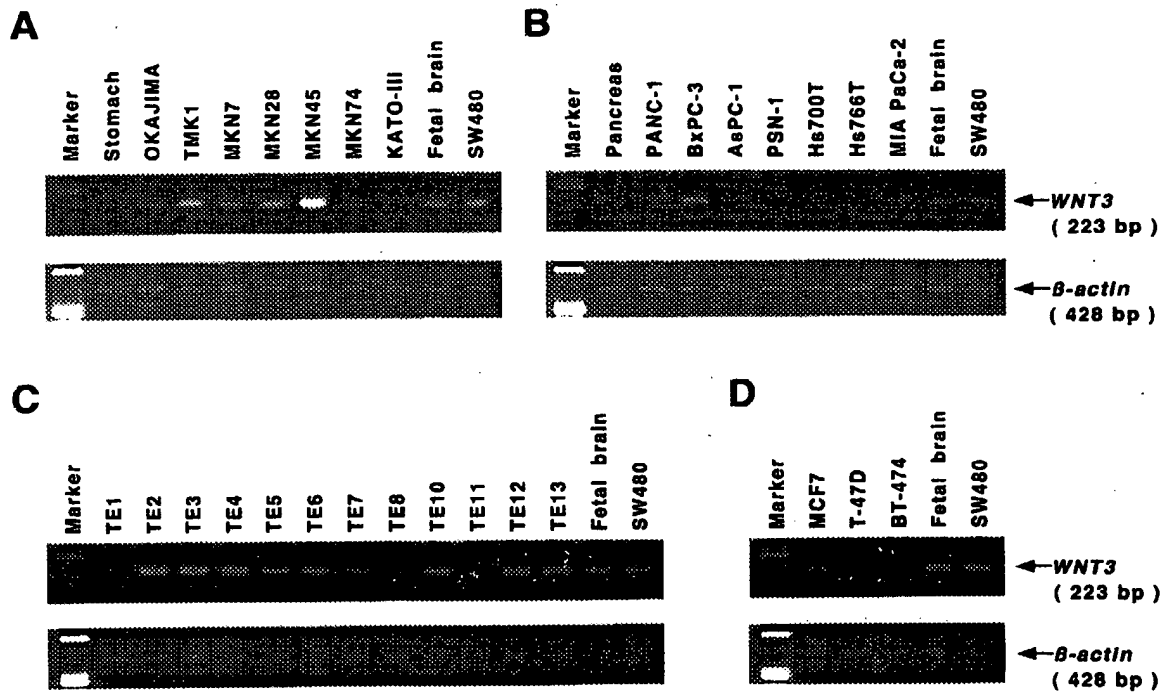

Figure 3. Expression of *WNT3* mRNA in human cancer cell lines. (A), Gastric cancer cell lines. (B), Pancreatic cancer cell lines. (C), Esophageal cancer cell lines. (D), Breast cancer cell lines. Ten ng of poly(A)$^+$ RNAs were used as templates for cDNA-PCR. *WNT3* cDNA (223 bp) was detected by 28 cycles of cDNA-PCR with PW3-03 and PW3-02 primers, and *β-actin* cDNA (428 bp) by 17 cycles of cDNA-PCR with BACT3 and BACT2 primers. *β-actin* cDNA was almost equally detected. Amount of *WNT3* cDNA was significantly higher in MKN45 cells (gastric cancer) than in fetal brain or SW480 cells.

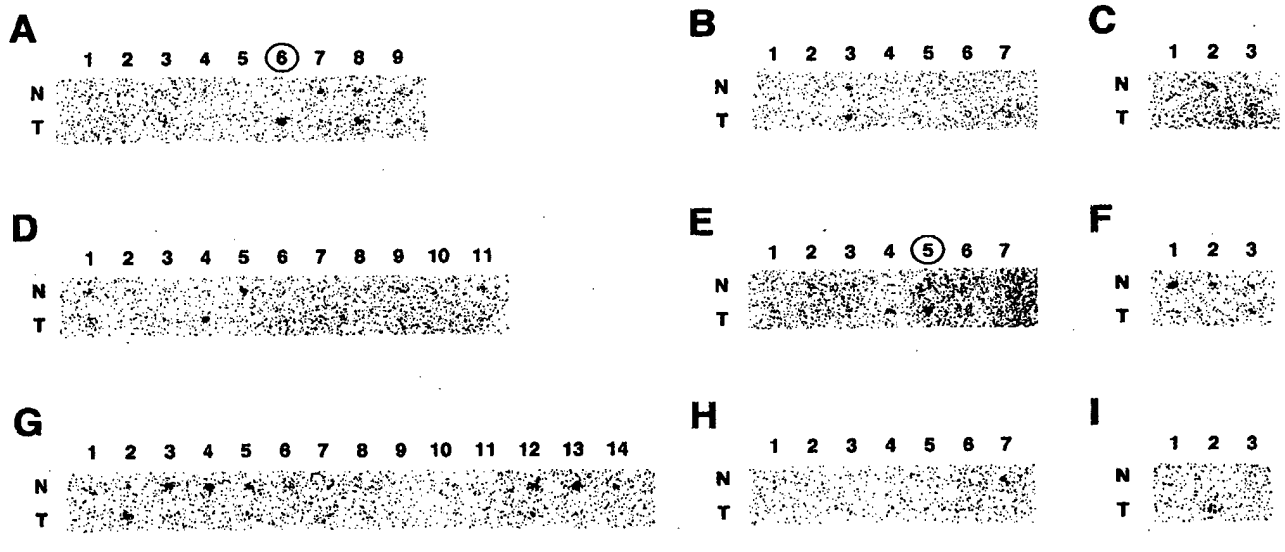

Figure 4. Expression of *WNT3* mRNA in various types of human primary cancers. (A), Breast cancer. (B), Gastric cancer. (C), Lung cancer. (D), Colon cancer. (E), Rectal cancer. (F), Prostate cancer. (G), Kidney tumors. (H), Uterus tumors. (I), Ovarian cancer. Matched tumor/normal expression array filter was hybridized with [α-^{32}P]dCTP-labeled W032 probe. *WNT3* was significantly up-regulated in a case of primary breast cancer and in a case of primary rectal cancer.

between *WNT3* and *WNT3A* genes. Intron 4 was identified in 3'-UTR of the *WNT3* gene, at the position 8 bases downstream of the stop codon, but not in the corresponding region of the *WNT3A* gene. Intron 4 might be inserted into the *WNT3* gene during or after duplication of an ancestral gene cluster.

WNT3 mRNAs of 1.6-, 3.4-, and 4.6-kb in size were detected by Northern blot analyses with the W302 probe (Fig. 2). *WNT3* ESTs, polyadenylated at the position 124 or 1951 bp downstream of the 3'-terminal end of *WNT3* cDNA, were predicted to correspond to 1.6- and 3.4-kb *WNT3*

mRNAs, respectively. Thus, at least 1.6- and 3.4-kb *WNT3* mRNAs might be generated due to alternative polyadenylation.

WNT3 mRNAs of 3.4- and 4.6-kb in size were faintly expressed in human fetal and adult brain (Fig. 2), which was consistent with mouse *Wnt-3* expression in embryos and in adult brain (22). Although expression of mouse *Wnt-3* in mouse testis is unclear, the 1.6-kb *WNT3* mRNA was weakly expressed in human testis (Fig. 2). Role of *WNT3* in spermatogenesis should be investigated in the future.

WNT3 mRNAs were relatively highly expressed in A549 cells (lung cancer) and MKN45 cells (gastric cancer) among 37 human cancer cell lines (Figs. 2C and 3). In addition, *WNT3* mRNA was significantly up-regulated in a case of primary breast cancer and in a case of primary rectal cancer among various types of human primary cancers (Fig. 4). Human WNT3 and mouse Wnt-3 shows 98.9% total-amino-acid identity, and overexpression of mouse *Wnt-3* induces mouse mammary carcinogenesis through activation of the WNT - ß-catenin - TCF signaling pathway (22,32). Thus, *WNT3* might play a key role in some cases of human breast, rectal, lung, and gastric cancer through activation of the WNT - ß-catenin - TCF signaling pathway, similar to mouse *Wnt-3*.

Acknowledgements

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OPINION

Wnts as morphogens? The view from the wing of *Drosophila*

Alfonso Martinez Arias

Morphogens are diffusible signalling molecules that pattern cellular fields by setting up differential gene expression in a concentration-dependent manner. Members of the Wnt family of signalling molecules are generally considered to be classical morphogens. However, a close analysis of their activity indicates that they do not fulfil all of the critera that are associated with the classical definition.

Pattern formation is a central issue in developmental biology. It refers to the process that leads to the spatial arrangement of different kinds of cells in ways that make sense either functionally (for example, the spacing of different sense organs or the scales on an epithelium, or the arrangement of different kinds of mesoderm in an early embryo) or visually (for example, the stripes of a zebra or the spots of a butterfly). From a mechanistic point of view, these patterns raise some important questions. How are such complex cellular arrangements specified? What are the molecules that provide the information for this process? The concept of morphogens (BOX 1), and in particular their linkage to the idea of positional information by Lewis Wolpert[1], provides a powerful, simple and elegant solution to the problem.

In its simplest definition, a morphogen is a diffusible molecule that elicits direct long-range concentration-dependent changes in gene expression and cellular behaviour[1–3]. This idea is seductive because it provides a simple correlation between specific substances (morphogens), an input (their concentration) and an output (the response of the cells, which is expressed as a pattern). The past ten years have bestowed the accolade of morphogen on several secreted molecules that are involved in cell interactions (BOX 1) and, in some cases, have led to proof-of-concept (for examples, see REF 3).

Members of the Wnt (Wingless/Int-1) family of signalling molecules have often been associated with the classical idea of a morphogen[2–3]. However, the case for these molecules might not be as clear cut as it is for other molecules such as Hedgehog (Hh) and bone morphogenetic proteins (BMPs). Here, I discuss the evidence that Wingless — a *Drosophila melanogaster* Wnt — is a candidate classical morphogen, which is a concept that came mainly from the study of Wingless activity in the patterning of the wing during larval development. I believe that the conclusion that Wnt is a classical morphogen has not taken into account important biological parameters of the system under study (for example, the order of the onset of gene expression and the growth of the wing). The consideration of these parameters raises the possibility that, in many instances, Wingless does not function as a classical inductive morphogen, and that its concentration-dependent responses have more to do with an important role for Wingless in modulating the effects of inductive molecules.

Wingless as a classical morphogen

Wingless is a founder member of the Wnt family of signalling molecules[5]. Its gene, *wingless* (*wg*), was first identified as a mutation, *wg*^{1} (REF 6), that removes the wing of *Drosophila*. *wg*^{1} is a regulatory mutation that

Box 1 | **Morphogens**

The key attributes of morphogens — on the basis of the classical definition and on present perceptions — are:

- They are secreted, diffusible molecules that come to be distributed in a concentration gradient from a fixed spatial source.
- They generate several (at least three) discrete cellular states in response to different thresholds of the concentration gradient. These cell states are usually associated with differential gene expression.
- They are instructive (that is, they function as a determinant of the cell state) in a direct manner (that is, without intermediates; the response of the cells does not depend on the cell changing states first).

Examples of molecules and situations that fulfil the criteria above are:

Spatzle, specifying the dorsoventral axis of the *Drosophila melanogaster* embryo.

Decapentaplegic, patterning the *Drosophila* wing.

Activins, specifying and patterning the mesoderm during the early development of amphibians and other vertebrates.

Bone morphogenetic proteins 2 and 4, mediating the activity of Spemann's organizer in *Xenopus laevis*.

Sonic Hedgehog, specifying motorneuron pools in the vertebrate central nervous system.

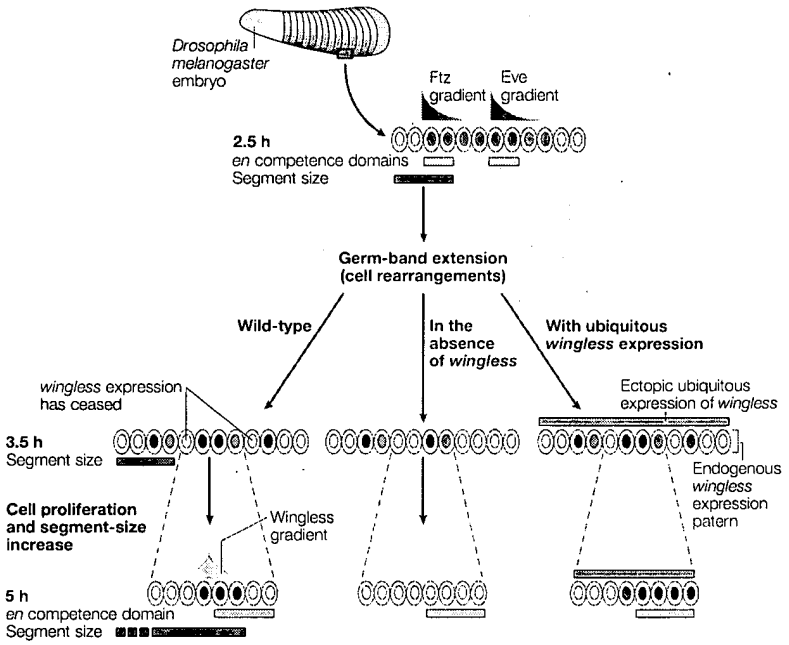
identified an essential function for Wingless in the initial specification of the wing[7], and null alleles of *wg* have more widespread effects on fly development[8].

The *wg* gene encodes a secreted protein that can function at a distance from its source[9,10]. In the *Drosophila* embryo, there is some evidence for concentration-dependent responses to Wingless. However, these responses are not multiple (that is, for a given pattern of Wingless expression, different concentrations do not elicit different responses) and there is no evidence that the establishment of these responses is a direct consequence of Wingless activity or that the concentration of Wingless is important for them[11,12]. The regulation of *engrailed* (*en*) expression in the *Drosophila* embryo provides an example of this. During the patterning of the blastoderm, the pair-rule genes — each of which specify a simple alternation with a repeat distance of two segments — establish domains of competence, in which defined groups of cells have the ability to express *en*[13] (BOX 2). However, *en* expression is only stabilized in a subset of these cells, that is, those that are exposed to 'enough' Wingless for a certain amount of time. The initiation of *en* expression is independent of Wingless, which only determines the maintenance of this expression[14]. The expression of *wg* is restricted to a one-cell-wide stripe per embryo segment and the extension of this expression to the whole of the segment does not result in a related expansion in the expression of *en*. Instead, *en* simply enlarges its domain slightly to occupy the region where the pair-rule genes activated its expression at the blastoderm stage[15] (BOX 2).

The specification of naked cuticle is a second example of Wingless function in the embryo[11,16]. In the absence of Wingless, the ventral epidermis develops a 'lawn' of denticles from anterior to posterior. In the wild type, however, periodically arranged sources of Wingless generate gaps in this lawn. The extent of these gaps is determined by the extent of Wingless diffusion and these gaps are achieved by repression of the gene *ovo/shaven-baby (svb)*[16]. As in the case of *en* expression, this repression and the ensuing patch of naked cuticle are the simple and unique response to a varying concentration of Wingless. So, for the repression of *ovo/svb*, as well as for *en* expression, all that is needed is Wingless signalling above a certain threshold, which is probably very low, and this is sufficient to elicit a unique response.

During larval development, Wingless has important functions in the development and patterning of the different elements of the adult fly. The legs and wings have been particularly well studied and it is in these contexts that the idea that Wingless is a classical morphogen has been proposed[17–19]. In particular, many of the arguments for Wingless as a classical morphogen are derived from the effects of Wingless on wing patterning. In the late third larval instar, which is the late phase of growth of the adult tissues (~100 h after egg laying), the developing wing is bisected by a narrow stripe of *wg* expression, which is the source of a steep symmetric gradient of the Wingless protein (FIG. 1a). This stripe of Wingless happens to coincide with a developmental landmark — the

Box 2 | **Initiation versus maintenance of *engrailed* expression**



The 'permissive' function of Wingless signalling is illustrated by its effect on the expression of *engrailed* (*en*) in the early *Drosophila melanogaster* embryo, which can be generalized to its effects on other targets. Early in the development of *Drosophila*, the embryo is subdivided into segments along the anterior/posterior axis by the transcription factors Fushi tarazu (Ftz; red in the figure) and Even skipped (Eve; blue). The genes *ftz* and *eve* are pair-rule genes. Ftz and Eve function as activators of *en* expression (light and dark brown shading in the figure highlight the levels of *en* expression (low and high expression, respectively)) and repressors of *wingless* (*wg*) expression (light and dark green shading in the figure highlight the levels of *wg* expression (low and high expression, respectively)), and they are deployed in alternative gradients that define regions of high and low activity across each segment. As a result of these patterns of expression, *en* and *wg* come to be expressed in adjacent single-row stripes of cells, which are determined by the highest (*en*) and lowest (*wg*) activity of Ftz and Eve. So, above a certain threshold, high levels of Ftz and Eve endow cells with the competence to express *en* (*en* competence domains are highlighted by yellow rectangles), perhaps by opening up chromatin and recruiting the basal transcriptional machinery to its promoter. Wingless signalling is necessary to make this pattern of gene expression stable.

As cells begin to proliferate and the segments begin to grow (3.5–5 h), Wingless diffuses from its source and creates a concentration gradient across the 'domains of competence' that were set up by Ftz and Eve. Above a certain threshold, Wingless maintains the expression of *en*, perhaps by stabilizing the structure of the chromatin. In the absence of Wingless, *en* expression is not compromised initially, but decays quickly. On the other hand, ectopic ubiquitous expression of *wingless* across the whole developing segment only allows *en* to be expressed in cells that are already competent to express it. This effect can be best seen when Wingless is ubiquitously provided in a *wg*-mutant embryo. In this case, the expression of *en* is identical to that of wild-type embryos in which *wg* is ubiquitously expressed. This experiment highlights the fact that, with regard to *en* expression, Wingless is a maintenance factor for the initial activity of the pair-rule genes.

appearance of the dorsoventral (DV) compartment boundary — and has therefore been endowed with organizing properties[18,19]. At this stage, and centered around the source of Wingless, there are three concentric domains of gene expression: a broad domain that expresses *vestigial* (*vg*); a narrower domain that expresses *Distal-less* (*Dll*); and a very narrow domain, which is adjacent to the source of Wingless, that expresses *achaete* (*ac*) (FIG.1b,c). Because alterations in the concentration of Wingless (for example, by the creation of ectopic sources in the developing wing) have subtle effects on the levels of expression of these genes and, in the case of *ac*, also on its domains of expression, it has been suggested that Wingless functions as a classical morphogen to pattern the wing[18,19]. As for any other putative classical morphogen, the most important element in the argument is that the three concentric domains of gene expression represent direct threshold responses to the gradient of Wingless. If this is not the case, Wingless cannot be said to function as a classical morphogen.

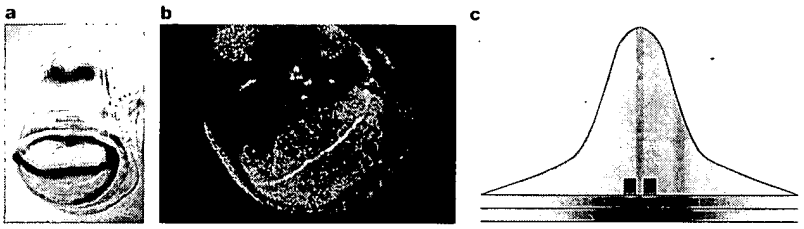


Figure 1 | **Wingless signalling in the wing primordium of a third larval instar wing disc of *Drosophila melanogaster*.** **a** | The pattern of *wingless* (*wg*) expression (blue) is shown by a β-galactosidase reporter that was inserted at the position of the *wg* gene. Notice a circle that delimits the wing tissue, and a stripe of expression that bisects this and delimits the dorsoventral (DV) boundary. **b** | The wing disc showing the expression of the Vestigial protein (red) relative to the source of Wingless at the DV boundary (yellow). **c** | A schematic of the gradient of the Wingless protein (green) and the observed patterns of response in the third larval instar wing. Achaete (black) is found in cells that are adjacent to the source of Wingless (dark green), and Distal-less (red) and Vestigial (blue) have similar graded responses. For further details, see REFS 18,19. The photograph in part **b** was kindly provided by S. Carroll and J. Paddock (University of Wisconsin, Madison, USA).

Wingless and the patterning of the wing

The patterns of expression of *vg*, *Dll* and *ac* in the third instar disc relative to the DV stripe of *wg* expression are indeed very indicative of the function of a classical morphogen. However, there are some problems with this interpretation because, throughout the development of the wing, *wg* expression is not fixed but undergoes several transitions between different patterns[7,20] (FIG. 2). Therefore, to evaluate the possibility that Wingless functions as a classical morphogen, it is important to consider the way the wing grows, the way the three genes are activated and, as the DV stripe of *wg* expression is only one of several patterns of *wg* expression during wing development, the relationship between the initial expression of each of these genes and the pattern of *wg* expression at that time. The consideration of these variables leads to a very different picture of how the domains of *vg*, *Dll* and *ac* expression arise and, more importantly, of the contribution of Wingless to this process[20–22].

The wing of *Drosophila* develops from a small group of cells that is specified by Wingless at the beginning of the second larval instar (~48 h after egg laying)[7,23]. From this moment onwards, a series of successive and mutually-dependent interactions between transcription factors and signalling molecules leads to the pattern that is visible in the third larval instar[4,20] (FIG. 1). In general, these interactions involve the activity of signalling centres along the anterior/posterior and the DV axes, and the activity of Wingless is associated with the DV axis.

At the onset of wing development, *wg* is expressed over the ventral side of the wing disc. From this moment, and until the third larval instar, it is expressed in a sequence of dynamic patterns that culminate in the formation of the DV stripe during the first half of the third instar (FIG. 2). By this time, both *vg* and *Dll* have been activated and are expressed in particular domains of the developing wing. The first gene to be activated is *vg*, early in the second instar, when *wg* is expressed in a rapid sequence of patterns that, for the most part, always overlap with the expression of *vg* (FIG. 2). At this stage, Wingless cannot elicit the ectopic expression of *vg*, but Delta, a ligand for the Notch receptor, can. Furthermore, when Wingless is provided together with Delta, it is possible to see a stronger effect of Delta, that is, the activity of Delta shows that there is a function of Wingless with which it works synergistically[21,22]. This led to the suggestion that the initiation of *vg* expression depends on the combination of Delta and Wingless, with Delta providing the initial input and Wingless providing a modulatory and stabilizing influence[21,22].

The expression of *Dll* is activated after that of *vg*, but it also occurs before *wg* is expressed in a symmetric DV stripe and at the time when *wg* is expressed in a very complex pattern over the developing wing. Again, Wingless is not able to elicit new expression of a target, in this case *Dll*. However, Vestigial can do this and, in a situation reminiscent to that of the earlier stage, Wingless functions synergistically, this time with Vestigial, to regulate the expression of *Dll*[21]. So, it would seem that, once activated, Vestigial induces the expression of *Dll*, the expression of which is only maintained in a domain that is determined by the activity of Wingless. Both of these situations are reminiscent of the regulation of *en* expression by Wingless in the embryo (BOX 2); a regulatory event (Delta signalling in the case of *vg* and Vestigial activity in the case of *Dll*) provides an input that defines a competence domain (as the pair-rule proteins do for *en*) and Wingless then stabilizes gene expression in a subdomain that is determined by its range of action and the responsiveness of the genes. By the time the DV stripe of Wingless appears, the expression of *vg* and *Dll* is well established and the function of Wingless is to modulate and maintain it with reference to the DV boundary.

At first sight, the case of *ac* seems different, because the onset of its expression is coincident with the DV stripe of *wg* expression. However, it might not be that different, because *ac* expression could be elicited by something other than Wingless (perhaps by Distal-less itself) and then be maintained where there are high levels of Wingless. It might well be that most of the wing is primed to express *ac*, and that only those regions that are exposed to high levels of Wingless do so stably. In the wild type, the Wingless gradient is very steep (FIG. 1c) and this, coupled to a very high response threshold, might be the simple explanation for the very narrow pattern of *ac* expression. This possibility is supported by observations of the function of Wingless in the regulation of *ac* in the notum — the main thoracic body part of the adult fly. In this case, ectopic expression of Wingless has little effect on the pattern; that is, Wingless can only elicit bristles, the associated sensory organs and *ac* expression where the 'pre-pattern' allows it to

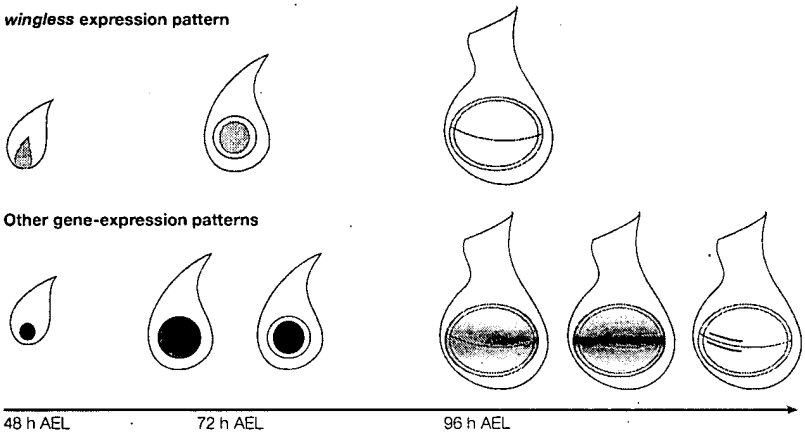


Figure 2 | **Temporal relationship between different patterns of *wingless* expression and the onset and modulation of gene expression during wing development.** During the second larval instar (about 48 h after egg laying (AEL)), *wingless* (*wg*; green) is expressed over the whole of the ventral region of the wing disc from which the wing primordium emerges, as identified by the expression of *vestigial* (*vg*) (blue). At the beginning of the third instar (about 72 h AEL), *wg* expression begins to be modulated by a pattern that occupies the whole of the wing primordium. At this time, *vg* is already expressed in this domain and *Distal-less* (*Dll*) expression (red) is initiated in a similar domain. By mid or late third instar (~96–100 h AEL), *wg* is expressed in the dorsoventral (DV) stripe and in a series of rings that outline the wing pouch. At this stage, the expression of *vg* and *Dll* is modulated with reference to the DV source of Wingless, and *achaete* expression (black) is initiated in cells that are adjacent to those producing Wingless. For further details of this process, see REFS 20–22.

do so[24,25]. Another important issue of the relationship between *ac* and Wingless is that only cells that are adjacent to the source of Wingless express *ac*[26]. This is not an effect at a distance.

Wingless is not a classical morphogen

An essential issue in assigning the qualities of a classical morphogen to a signalling molecule is the requirement for the candidate molecule to induce gene expression over a distance directly and *de novo*, that is, to be instructive[1–3]. The observations and experiments summarized above (also see REF. 22) indicate that Wingless does not initiate the expression of *vg* or *Dll* and this is supported by some experiments that tested this point directly[20,21].

The case for Wingless as a classical morphogen rests on the assumption that it is the diffusion of Wingless from the DV stripe that directly establishes the patterns of *vg*, *Dll* and *ac* expression. In the best of cases, it is difficult to devise experiments that test whether a molecule fulfils the criteria of a classical morphogen or not. The situation of the wing of *Drosophila* is particularly complex, because not only is there always an endogenous source of the molecule being tested, which complicates the analysis of the responses, but this source has a dynamic pattern of expression. The latter point is important because it means that it is always difficult to be sure which pattern of Wingless is responsible for a particular effect.

An approach that can be used to get around this problem is to take a mutant in which the DV stripe of *wg* is missing, and to look at the effects of reintroducing Wingless under these circumstances. This experimental situation is provided by wing discs with impaired Notch signalling. Mutants for *Suppressor of Hairless* or *apterous* lack the DV stripe of *wg*, as well as expression of *vg*, *Dll* or *ac*. Furthermore, these mutants lack wings. So, based on the assumption that Wingless functions as a classical morphogen to mediate the development and patterning of the wing, reintroducing Wingless to these mutants should, at the very least, restore wing development. However, this was not found to be the case[20,21], which confirms the impression that is gained from other studies, which is that Wingless cannot trigger the expression of the genes that mediate wing development and patterning. Interestingly, expression of *vg* in these mutants can rescue wing development[21], which underscores the fact that something else, and not Wingless, triggers the expression of *vg*. This result indicates that Wingless is not essential for wing development and also argues against the idea that it functions as a classical morphogen.

> "...although it is not possible to say that Wingless functions as a morphogen in the classical sense, its concentration-dependent effects on some aspects of gene expression highlight the need to reconsider the idea of a morphogen."

Cell fate: initiation or maintenance?

The idea that Wingless functions as a classical morphogen during wing development has influenced our way of looking at other Wnt proteins. The essence of a classical morphogen is its ability to induce different cell states in a concentration-dependent, direct and instructive manner. If any of these criteria are not met, the candidate should not be deemed a classical morphogen. During the patterning of the wing, Wingless fails to meet these criteria (also see REF. 22). However, one property of morphogens — that is, the concentration-dependent responses — should be looked at carefully. In the classical definition, this parameter determines the initiation of gene expression. In the context of Wingless signalling, it is clear that the maintenance, rather than the initiation, of the expression of different genes is sensitive to the concentration of Wingless and this might well be a general feature of Wnt-protein function (for examples, see REFS 21,22,27; see REF. 28 for an example from vertebrates).

So, although it is not possible to say that Wingless functions as a morphogen in the classical sense, its concentration-dependent effects on some aspects of gene expression highlight the need to reconsider the idea of a morphogen. It might be necessary to distinguish between functionally different kinds of morphogens (FIG. 3). The first kind of morphogen is the classical one, which fulfils the strict definition and includes, for example, BMPs and members of the Hh family[3]. The second kind of morphogen would encompass molecules that, rather than being instructive, have a secondary, but essential, role in the maintenance of cell fates. Wingless is the model example of this class. In all the cases that have been analysed in detail, the absence of Wingless does not affect the initial adoption of a fate, but affects only its maintenance and stability

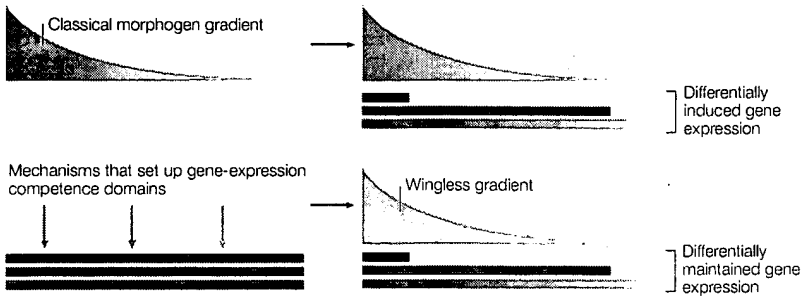


Figure 3 | **Different outcomes of concentration-dependent initiation or maintenance of gene expression. a** | A classical morphogen (pink) elicits differential gene expression (represented by the black, red and blue bars) by activating different genes at different concentrations. **b** | Molecules like Wingless (green), and possibly other Wnt proteins, function by modulating gene expression in a concentration-dependent manner. Genes with broad domains of potential expression are set up by various mechanisms (as indicated by the coloured arrows), which include the genetic history of the cell or intercellular signalling. Each of these genes has a different response threshold to Wingless. So, a gradient of Wingless results in the stable differential expression of the different genes and the patterning of the field of cells simply by determining their domains of maintenance. One way in which this can be achieved is by modulating chromatin structure.

(for a review, see REF. 22). The differential response of genes to this maintenance function results in a pattern. For example, there is very little difference between the domains of *Dll* and *vg* expression, and whatever difference there is might be due to subtly different responses to the maintenance activity of Wingless.

Conclusions and perspectives

One problem with the idea of a classical morphogen is that it lacks a well-defined mechanistic element. The original concept was formulated at a time when little was known about the molecular mechanisms of pattern formation and was, out of necessity, vague. Perhaps this is the reason why we often encounter hair-splitting debates on whether something is or is not a classical morphogen. For example, does Sonic hedgehog function as a classical morphogen during the patterning of the vertebrate limb? In the course of time, the original concept of a classical morphogen has evolved to accommodate molecules that are involved in pattern formation at a distance, but it might have to evolve more and become more precise if we do not want to become arbitrary in what we call, in the classical sense, a morphogen.

The distinction between two kinds of morphogens in terms of their molecular function might not just be an issue of semantics. The initiation and maintenance of gene expression are two mechanistically different steps[27,29] that have to be linked for cells to adopt fates stably. Classical morphogens are one way of activating the first step[1–4], and Wnt molecules might be a way of regulating the second step 'from the outside'. It is well known that chromatin-remodelling proteins have a role in stabilizing the gene expression that is associated with cell fates; Wnt signalling might be a way of regulating this process through intercellular signalling. The effects of molecules such as Wnt proteins therefore sharpen and refine the more 'coarse-grain' patterns that are laid out by classical morphogens or, in other circumstances, by lineage-related mechanisms[21,22,27]. It will be interesting to see if, in the complex context of growing fields of cells (such as the wing discs), the classical concept of a morphogen can be sustained, or whether, from a mechanistic point of view, allowances will need to be made for the different roles of other signalling molecules.

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Dorsalizing signal *Wnt*-7a required for normal polarity of D–V and A–P axes of mouse limb

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FORMATION of the vertebrate limb requires specification of cell position along three axes[1]. Proximal–distal identity is regulated by the apical ectodermal ridge (AER) at the distal tip of the growing limb[2–6]. Anterior–posterior identity is controlled by signals from the zone of polarizing activity (ZPA) within the posterior limb mesenchyme[7–9]. Dorsal–ventral identity is regulated by ectodermally derived signals[10–14]. Recent studies have begun to identify signalling molecules that may mediate these patterning activities. Members of the fibroblast growth factor (FGF) family are expressed in the AER and can mimic its proximal–distal signalling activity[15,16]. Similarly, the gene *Sonic hedgehog* (*Shh*) is expressed in the ZPA, and *Shh*-expressing cells, like ZPA cells, can cause digit duplications when transplanted to the anterior limb margin[17,18]. In contrast, no signal has yet been identified for the dorsal–ventral axis, although *Wnt*-7a is expressed in the dorsal ectoderm, suggesting that it may play such a role[19,20]. To test this possibility, we have generated mice lacking *Wnt*-7a activity. The limb mesoderm of these mice shows dorsal-to-ventral transformations of cell fate, indicating that *Wnt*-7a is a dorsalizing signal. Many mutant mice also lack posterior digits, demonstrating that *Wnt*-7a is also required for anterior–posterior patterning. We propose that normal limb development requires interactions between the signalling systems for these two axes.

Wnt-7a is expressed in the flanking ectoderm of the trunk prior to limb bud outgrowth. At 8.75 days postcoitum (d.p.c.), *Wnt*-7a expression encompasses the presumptive forelimb region, extending from the level of the last few somites into the ectoderm overlying the presomitic mesoderm (Fig. 1*a*). At the level of the presumptive hindlimb, ectodermal expression is first apparent at 9.25 d.p.c. (Fig. 1*a*). During initial stages of limb-bud outgrowth (9.25 d.p.c., forelimb; 9.75 d.p.c., hindlimb) and patterning, *Wnt*-7a transcripts are uniformly distributed throughout the dorsal limb ectoderm (Fig. 1*a*).

To investigate the function of *Wnt*-7a, we used gene targeting in mouse embryonic stem (ES) cells to generate a likely null allele (Fig. 1*b–d*). Matings between mice heterozygous for the targeted allele produced homozygous offspring at expected mendelian frequencies (+/+, 32; +/−, 70; −/−, 37). Although apparently fully viable, *Wnt*-7a$^{-/-}$ mice have limb abnormalities and are also sterile.

Ventral structures develop normally in *Wnt*-7a$^{-/-}$ limbs, but many dorsal tissues adopt ventral fates. Distal limb structures are particularly useful in distinguishing dorsal from ventral cell types (Figs 2, 3). The ventral skin of wild-type digits is devoid of fur, lacks pigmentation and exhibits a prominent set of transverse striations (Fig. 2*a*). Moreover, dermal footpads protrude at the base of the digits and at the distal tips of the digits (Fig. 2*a*). Normal flexion of the digits depends upon a specific arrangement of ventral tendons projecting to a series of small, ventrally located, sesamoid bones. In contrast, the dorsal half of the limb is pigmented, covered in hair, and does not possess footpads or sesamoid bones (Fig. 2*b*). The major dorsal tendons travel along the outside edges of the digits (Fig. 3*b*), while a larger tendon runs down the middle of the ventral surface (Fig. 3*a*). Dorsal-to-ventral transformations of mesenchyme cell fate are observed in the footpads, skin, tendons, sesamoid bones and joints of *Wnt*-7a mutant limbs.

The most striking superficial abnormalities on the dorsal surface of mutant limbs are dermal thickenings representing duplications of the footpads normally found ventrally (Fig. 2*c, e, f*). The dorsal pads are visible as early as 15.5 d.p.c. (Figs 2*g–i*, 3*d, e*) and express *Pax*-9 (Fig. 2*i*), a putative transcription factor, normally restricted to developing ventral pads (A. Neubuser and R. Balling, personal communication). Ectopic dorsal pads are pigmented, as migration of melanocytes is apparently unaffected by the *Wnt*-7a mutation.

The dorsal surfaces of the digits in severe *Wnt*-7a mutants also lose hair and acquire noticeable striations, resembling the normal ventral surface (Fig. 2*c*). The growth of nails at the dorsal–ventral interface of the digits is truncated to a variable extent (Fig. 2*c, f*), and abnormal, pigmented thickenings, which probably represent duplications of the large ventral pads found at the distal ends of the digits (Fig. 2*a*), often grow over the rudimentary nails (Fig. 2*f*).

Alterations in dorsal–ventral polarity are also apparent in the tendons and bones. In adult *Wnt*-7a mutant limbs (Fig. 3*c*), a large dorsal tendon, closely resembling the central ventral tendons (Fig. 3*a*), projects distally along each digit. These duplications of ventral tendons are clearly visible in sections through 15.5 d.p.c. mutant limbs, where the dorsal tendons appear to be mirror images of ventral tendons (compare Fig. 3*d, f* with *e, g*).

Normal flexion of the digits requires the correct arrangement of tendons and their attachment to sesamoid processes on the ventral surface of the distal limb bones. Sesamoid bones appear in their normal ventral positions in *Wnt*-7a mutants (Fig. 3*i*), but paired sesamoid processes also develop in the dorsal half of mutant paws (Fig. 3*k, n, o*). Not surprisingly, *Wnt*-7a mutant limbs show abnormal flexion due to these alterations in dorsal–ventral polarity. In contrast to the flexion of wild-type digits (Fig. 3*l*), mutant digits are generally straight (Fig. 3*n*), presumably because the duplication of ventral tendons dorsally results in similar tendons exerting counterbalancing forces on either side of the joints. In addition, the paws of mutants usually splay downwards and outwards, indicating incorrect bending at both the wrist and elbow joints (Fig. 3*p, q*).

Although we consistently observe a ventralization of the dorsal limb, the phenotype is clearly more severe distally than proximally. Interestingly, experiments in the chick indicate that ectodermal signalling only regulates dorsal–ventral patterning in the distal limb[13,14]. Thus, whereas *Wnt*-7a is important in dorsal–ventral patterning, it is likely that other mechanisms are also involved.

To determine whether changes in ectodermal cell fate contribute to the observed phenotype, we examined the expression of a number of regionally restricted ectodermal markers between 9.5 and 10.5 d.p.c. *Wnt*-7a transcripts can be detected in the dorsal ectoderm of *Wnt*-7a$^{-/-}$ limbs (data not shown), sug-

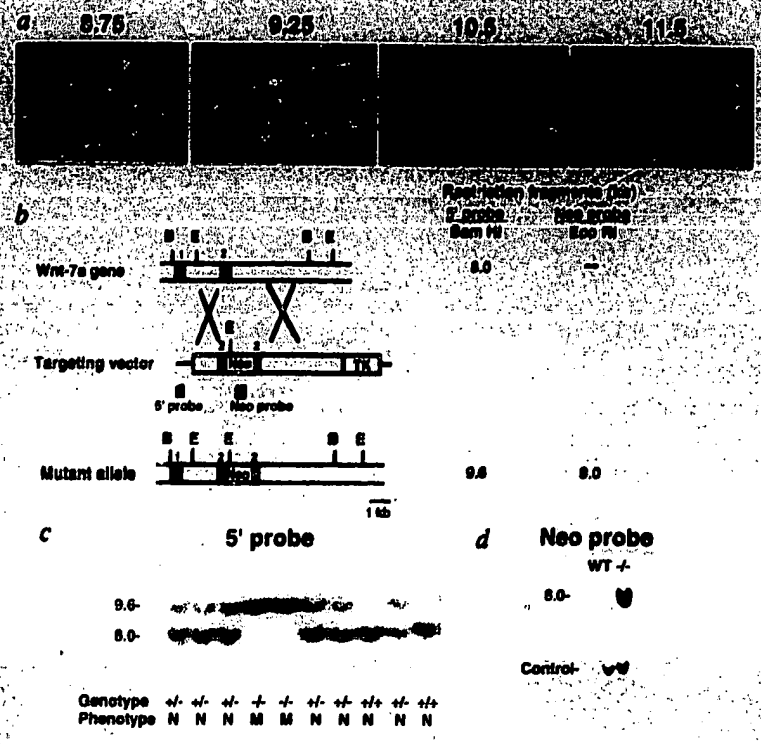

FIG. 1 *a*, Whole-mount *in situ* hybridization analysis of *Wnt*-7a expression from 8.75 d.p.c. to 11.5 d.p.c. *Wnt*-7a is expressed in the flank ectoderm (between the arrows) overlying the last few somites and extending into the presomitic region at 8.75 d.p.c., in the ectoderm overlying the presumptive limb bud regions at 9.25 d.p.c. (arrows), and uniformly throughout the dorsal ectoderm at 10.5–11.5 d.p.c. (dorsal views; d., dorsal; v., ventral). *b*, Targeting strategy to mutate the *Wnt*-7a gene by inserting a neomycin-resistance (*neo*) gene into the second exon. *c*, *d*, Southern blots demonstrating correct targeting of the *Wnt*-7a locus and genotyping of progeny (N, normal; M, mutant) using 5′ and *neo* probes indicated in *b*.
METHODS. Whole-mount *in situ* hybridizations were done as previously described[19]. *Wnt*-7a genomic clones were isolated from a 129/Sv library (Stratagene) using a cDNA probe spanning the entire coding region. A double-selection gene-replacement construct was designed to insert a *neo* gene into an *Nae*I site in the second exon of the *Wnt*-7a gene. *Wnt*-7a transcripts will be detected in mutant mice carrying this allele. The construct was linearized with *Sal*I and electroporated into CJ7 ES cells[31]. 96 G418/FIAU resistant colonies were picked and screened by Southern blot analysis. Filters hybridized with the *neo* probe were subsequently reprobed with a control *Wnt*-7a fragment that recognized both the wild-type and targeted alleles. Two independent targeting events were identified, and the targeted clones were injected into C57Bl/6J blastocysts to generate chimaeric mice. Tail or yolk sac DNA samples from offspring were initially genotyped by the same Southern protocol used to identify targeted clones. A PCR strategy was later devised to distinguish between targeted and wild-type alleles.

gesting that the dorsal ectoderm itself is not ventralized. Similarly, expression of *En*-1, *Bmp*-2, and *Dlx*-2, normally restricted to the ventral ectoderm[21–23], remains ventral in mutant limb buds (data not shown). Thus, cell fate changes would appear to be restricted to the underlying dorsal mesenchyme. These results also suggest that if a second ectodermal signal is required to specify ventral mesenchyme development, it is already present in the dorsal ectoderm, but the ventralizing activity is normally blocked at some regulatory level by *Wnt*-7a. Such a ventralizing signal, presumably acting on cells in the progress zone, may be widely expressed in the ectoderm or restricted to the dorsal–ventral interface in the region of the AER.

Wnt-7a also appears to regulate patterning along the anterior–posterior axis. There is a notable loss of posterior skeletal elements in mutant mice (Fig. 4*a*), even though *Wnt*-7a expression appears to be uniform throughout the dorsal ectoderm. Digit five, the most posterior digit, and the ulna, the more postcrior zeugopod element of the forearm, are often missing or abnormal in *Wnt*-7a homozygotes. The frequency of digit abnormalities increases in a graded fashion from anterior to posterior (Fig. 4*b*), as does the severity of defects within any individual limb. The extent of posterior abnormalities is quite variable (Fig. 4*b*), although part of the variability may result from the non-uniform genetic background of the animals. Loss of posterior bones is

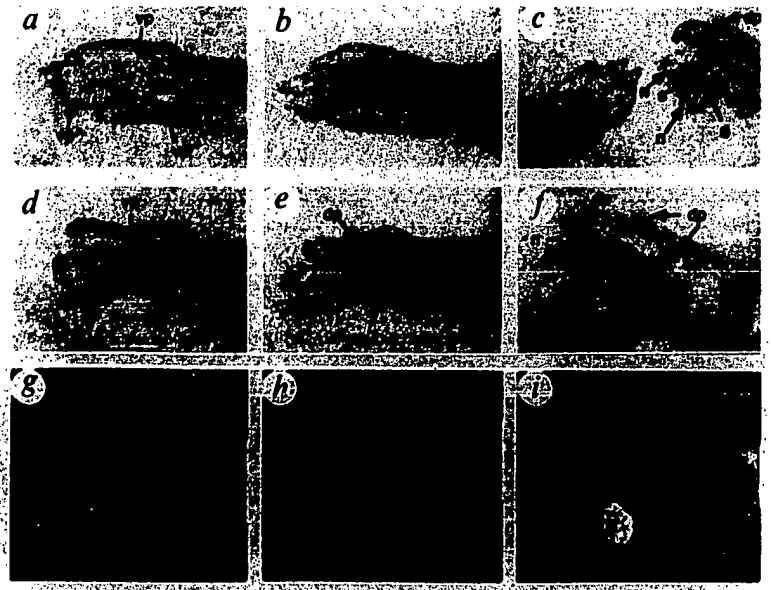

FIG. 2 Superficial views of *Wnt*-7a mutant limbs demonstrating the duplication of dermal pads on the dorsal surface of the paws. *a*, *d*, Ventral surface of wild-type (*a*) and mutant (*d*) forelimbs two weeks after birth showing the normal ventral pads (vp). *b*, *e*, Dorsal surface of wild-type (*b*) and mutant (*e*) forelimbs two weeks after birth showing ectopic dorsal pads (dp) on the mutant limbs. *c*, Wild-type and mutant adult forelimbs. Note the duplicated dorsal footpads (dp), the striations and lack of hair on the skin (s), and the abnormal nails (n) of the mutant limb. *f*, Close-up of adult mutant forelimb. The ectopic dorsal footpads (dp) maintain the same anterior–posterior and proximal–distal positions as the normal ventral pads (vp). The mutant nails (n) are truncated and overgrown by a duplication of the distal footpads. *g–i*, Whole-mount *in situ* hybridization demonstrating *Pax*-9 expression in developing pads of 15.5-d.p.c. embryos. *g*, Wild-type ventral pads (vp) express *Pax*-9. *h*, No *Pax*-9 expression is apparent on the dorsal surface of wild-type limbs. *i*, The dorsal surface of *Wnt*-7a homozygous limb buds shows ectopic *Pax*-9 expression in developing dorsal pads (dp).

not accompanied by any apparent transf rmation of posterior structures towards an anterior phenotype.

The loss f posterior skeletal elements in mutant limbs suggests that *Wnt*-7a signalling may be necessary for n rmal ZPA function. Attenuation of ZPA activity, either by grafting fracti ns of the ZPA r irradiation of n rmal ZPA cells, also leads to the preferential loss of posterior skeletal structures[9,24]. One potential target of *Wnt*-7a activity is *Shh*, which is expressed in the posterior mesenchyme and appears to mediate ZPA function[18,25]. *Wnt*-7a expression in the forelimb bud precedes *Shh* expression, which is not visible until 9.75 d.p.c.[17]. Wild-type *Shh* expression in the posterior mesenchyme is strongest in the dorsal half of the limb bud (Fig. 4*c*), suggesting that a dorsally localized factor may regulate activity of the ZPA. Initial expression of *Shh* in the forelimb bud is detected at the normal time in *Wnt*-7a mutants (not shown), so that activation of the *Shh* gene does not depend upon *Wnt*-7a function. However, by 10.5 d.p.c. there are fewer *Shh*-expressing cells in the limb buds of most mutant embryos (Fig. 4*d*, *e*). The decrease in *Shh* expression at a given stage varies from embryo to embryo, resembling the variable loss of posterior skeletal elements observed in older animals and suggesting that the levels of *Shh* activity may regulate anterior–posterior polarity. Thus, animals with the l west levels f *Shh* expression in the early limb bud may subsequently develop limbs with the most severe posterior truncations. BMP-2, a member of the transf rming growth factor TGF-β family, is also expressed in the posterior mesenchyme at 10.5 d.p.c.[21]. Like *Shh*, *Bmp*-2 expression in the ZPA is mainly dorsal and is also lost to a variable extent in *Wnt*-7a mutants (data not shown). Thus, *Wnt*-7a activity is necessary fo- the establishment and/or maintenance of normal patterns of ZPA gene expression.

The available evidence suggest- that reciprocal signalling occurs between the ZPA and the AER; in particular, FGF-4 in the posterior AER and *Shh* in the ZPA are required to maintain each other's expression[26–29]. We detect a variable loss of *Fgf*-4 expression in *Wnt*-7a$^{-/-}$ limbs that parallels the loss of *Shh* expression in the ZPA (Fig. 4*f*, *g*). The strongest remnants of *Fgf*-4 expression are found at the posterior margin of the normal *Fgf*-4 domain, adjacent to the site of residual *Shh* expression. In

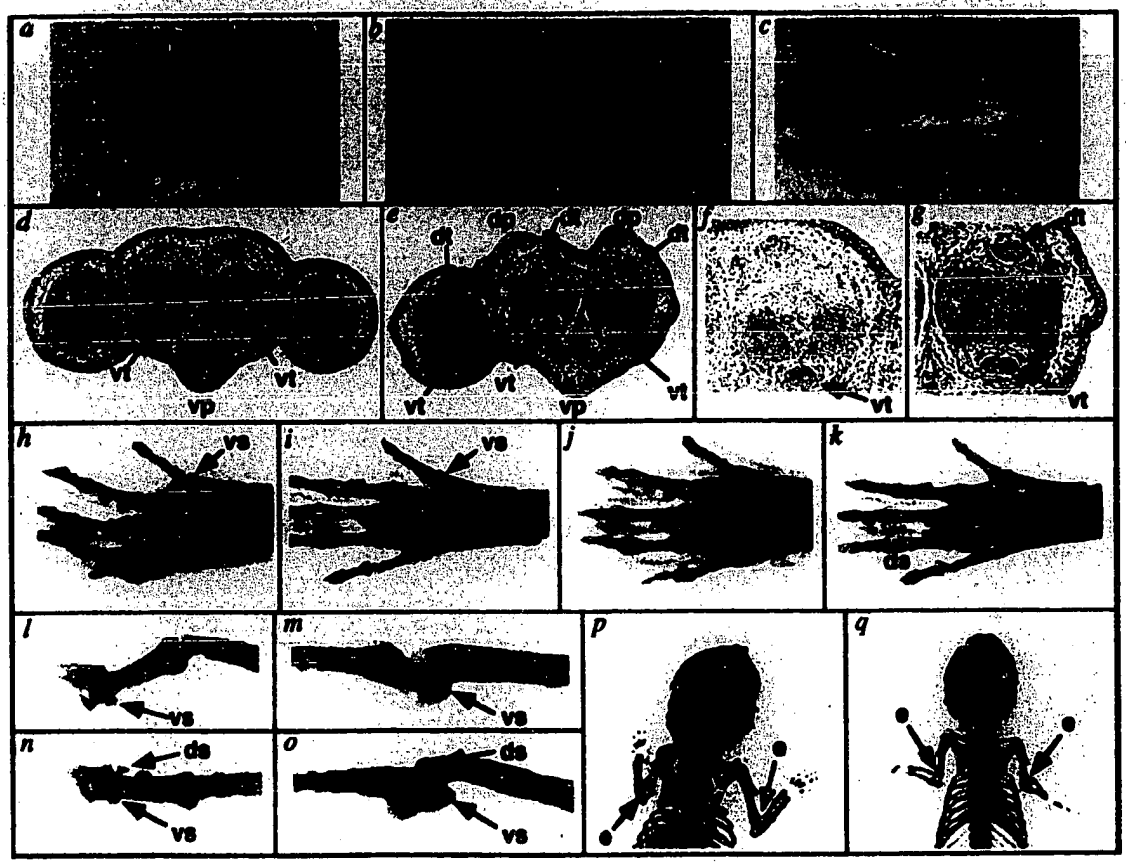

FIG. 3 Dorsal to ventral transformations of cell fate in *Wnt*-7a mutant limbs. *a–c*, Adult tendons showing wild-type ventral (a) and dorsal (*b*) and mutant dorsal (c) patterns. The dorsal tendons (dt) of the mutant limbs closely resemble normal ventral tendons (vt). *d–g*, Histological sections stained with haematoxylin and eosin through 15.5 d.p.c. forelimbs of normal (*d*, *f*) and mutant (*e*, *g*) embryos. The ventral footpads (vp in *d*) are duplicated on the dorsal half of mutant limbs (panel *e*). *f*, *g*, Higher magnification views of digit 4 from the same sections illustrate the duplication of ventral tendons (vt) in the dorsal half of the mutant limbs (dt). *h–k*, Sesamoid bones (vs) are present at the end of the metacarpals in the ventral half of wild type (*h*) and mutant (*i*) forelimbs. No dorsal sesamoids (ds) are present in wild-type forelimbs (*j*), but ectopic dorsal sesamoids are observed in mutant limbs (*k*). *l–o*, Higher-magnification view illustrating duplication of sesamoids at the distal end of the phalanges (*n*) and metacarpals (*o*) of mutant limbs compared to wild type (*l*, *m*). Note the decreased flexion of mutant digits (*n*) compared to wild type (*l*). *p*, *q*, Abnormal flexion of mutant forelimbs of neonates (*q*) relative to wild type (*p*) at the wrist and elbow (*e*) joints.

METHODS. The skeletons of newborn pups were fixed and stained by a modification of previous procedures. Pups were skinned, eviscerated, fixed in ethanol overnight, and incubated in acetone for 24 h. They were stained in 85 ml 70% ethanol, 5 ml glacial acetic acid, 5 ml 0.3% alcian blue in 70% ethanol and 5 ml 0.1% alizarin red in 95% ethanol for 4–6 h at 37 °C, followed by 3 days at room temperature. The skeletons were cleared in 1% KOH for 1 day, then passed through a glycerol series (20, 50, 80, and 100%) of variable duration. The length of individual steps in the protocol was increased for older animals.

b

	Forelimbs								Hindlimbs			
	L				R				L		R	
Digit	5	4	3	2	5	4	3	2	5	4	5	4
Deleted or abnormal	68	24	2	0	76	44	17	5	34	0	17	0
Deleted	54	0	0	0	56	0	0	0	7	0	2	0

	Ulna	Radius	Ulna	Radius	Fibula	Tibia	Fibula	Tibia
Deleted or abnormal	37	17	51	34	5	5	2	0
Deleted	7	0	17	0	0	0	0	0

FIG. 4 *Wnt*-7a homozygotes preferentially lose posterior skeletal elements. *a*, Skeletal preparations of neonatal wild-type and mutant forelimbs demonstrate the loss or reduction of posterior digits and the ulna and a range of observed phenotypes. S, scapula; H, humerus; R, radius; U, ulna; 1–5, digits 1–5. *b*, Tabulation of the loss of skeletal structures. Numbers represent precentage of abnormal development in postnatal *Wnt*-7a mutant limbs ($n = 41$). Skeletal preparations of mutant limbs were compared to wild type and scored as abnormal if they fell outside the range observed in wild-type specimens. *c–g*, Changes in *Shh* and *Fgf*-4 gene expression in *Wnt*-7a mutant forelimbs at 10.25 d.p.c. *c–e*, Posterior view of *Shh* expression in wild-type (*c*) and mutant (*d*, *e*) limb buds. Wild-type *Shh* expression is stronger in the dorsal half of the posterior mesenchyme (arrows denote the dorsal–ventral midline; d, dorsal; v, ventral). *Shh* expression in mutant embryos (arrows) is substantially decreased throughout the ZPA region. *f*, *g*, *Fgf*-4 expression in wild-type (*f*) and mutant (*g*) limb buds demonstrates that residual *Fgf*-4 expression in mutants is concentrated at the posterior margin of the normal expression domain (arrow).

contrast, residual *Shh* expression in mutants is not localized near the *Fgf*-4 domain, but is lost uniformly throughout the ZPA. Thus, *Wnt*-7a may directly regulate *Shh* expression, and the loss of *Fgf*-4 may be secondary to the loss of *Shh*. In fact, *Wnt*-7a is capable of inducing *Shh* expression in the limb under experimental conditions[30].

Our results show that *Wnt*-7a is important in regulating both dorsal–ventral and anterior–posterior polarity in the vertebrate limb, supporting the view that limb patterning is regulated by interactions among a network of signalling factors[28,29]. A major challenge will be to determine the mechanisms that integrate information encoded by these various signals to produce the appropriate three-dimensional structures for particular positions in the vertebrate limb. □

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RAPID COMMUNICATION

Induction of Kidney Epithelial Morphogenesis by Cells Expressing *Wnt-1*

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During kidney development, unknown signals derived from the ureteric bud induce metanephric mesenchymal cells to differentiate into nephron epithelia. In addition to the ureteric bud, a number of other tissues can act as heterologous inducers of this process *in vitro*, including embryonic spinal cord. In this report we demonstrate that *Wnt-1*, a gene that encodes a secreted glycoprotein expressed in embryonic spinal cord, is capable of conferring nephron-inducing activity to fibroblast cell lines. When cocultured with cells expressing exogenous *Wnt-1*, metanephric mesenchyme differentiated into glomerular and renal tubular epithelia. No such effect was observed using control cells. These data imply that the ability of embryonic spinal cord to act as an inducer of nephrogenesis may result from its production of Wnt-1 protein and suggest that a member of the *Wnt* gene family may be a mediator of renal epithelial morphogenesis *in vivo*.

The functional units of the metanephric kidney are the nephrons, which are composed of glomerular and tubular segments lined by diverse epithelial cell types. During embryogenesis, nephron formation is dependent on inductive interactions between cells of the metanephric blastema and ureteric bud, the two embryonic primordia that give rise to the kidney (Saxen, 1987). The ureteric bud induces mesenchymal cells of the metanephric blastema to differentiate into nephron epithelia. An organ culture model system of this process has previously been established in which isolated metanephric blastemata cells undergo apoptosis unless supplied with an inductive stimulus that permits them to differentiate into nephron epithelia (Grobstein, 1953; Koseki *et al.*, 1992). When cocultured with a suitable inducer tissue, metanephric blastema cells proliferate, aggregate, undergo a mesenchymal to epithelial transition, and form nephrons composed of glomerular and tubular epithelia (Saxen, 1987). In addition to ureteric bud, a limited number of other tissues can act as heterologous inducers of nephrogenesis *in vitro*, including embryonic brain and spinal cord (Grobstein, 1953, 1955; Saxen, 1987). This indicates that factors capable of initiating nephrogenesis are present in other embryonic tissues. However, a wide range of growth and differentiation factors implicated in the development of other embryonic organ systems have been found insufficient to induce renal differentiation *in vitro* (Weller *et al.*, 1993). Thus, although induction-dependent nephrogenesis has been extensively characterized over the past 40 years, the specific signals triggering this process have remained obscure.

The *Wnt* genes constitute a large family of genes encoding secreted intercellular signaling factors that are selectively expressed in a wide variety of tissues, including the kidney (Nusse and Varmus, 1992; Gavin *et al.*, 1990). The best characterized member of this family, *Wnt-1*, is expressed in the embryonic brain and spinal cord, both of which are known inducers of nephrogenesis *in vitro* (Nusse and Varmus, 1992; Saxen, 1987; Grobstein, 1953, 1955). Mouse *Wnt-1* is required for correct development of the fetal brain, while its homolog in *Drosophila*, the segment polarity gene *wingless*, acts in local intercellular signaling to modulate cell phenotype and developmental fate in the embryo and imaginal discs (Nusse and Varmus, 1992).

To investigate the potential role of *Wnt* genes in renal epithelial morphogenesis, metanephric blastemata were isolated from Gestation Day 13 rat embryos and cultured on polycarbonate filters. When cultured in isolation, such blastemata failed to differentiate. As previously demonstrated, however, blastemata cultured for 5 days with embryonic spinal cord on the opposite surface of the filter exhibited extensive nephron formation (Grobstein, 1953, 1955) (Figs. 1A–1D). Immunoblot analysis of embryonic spinal cord fragments showed that Wnt-1 protein is expressed in this tissue and that the predominant form comigrates with the 44-kDa species

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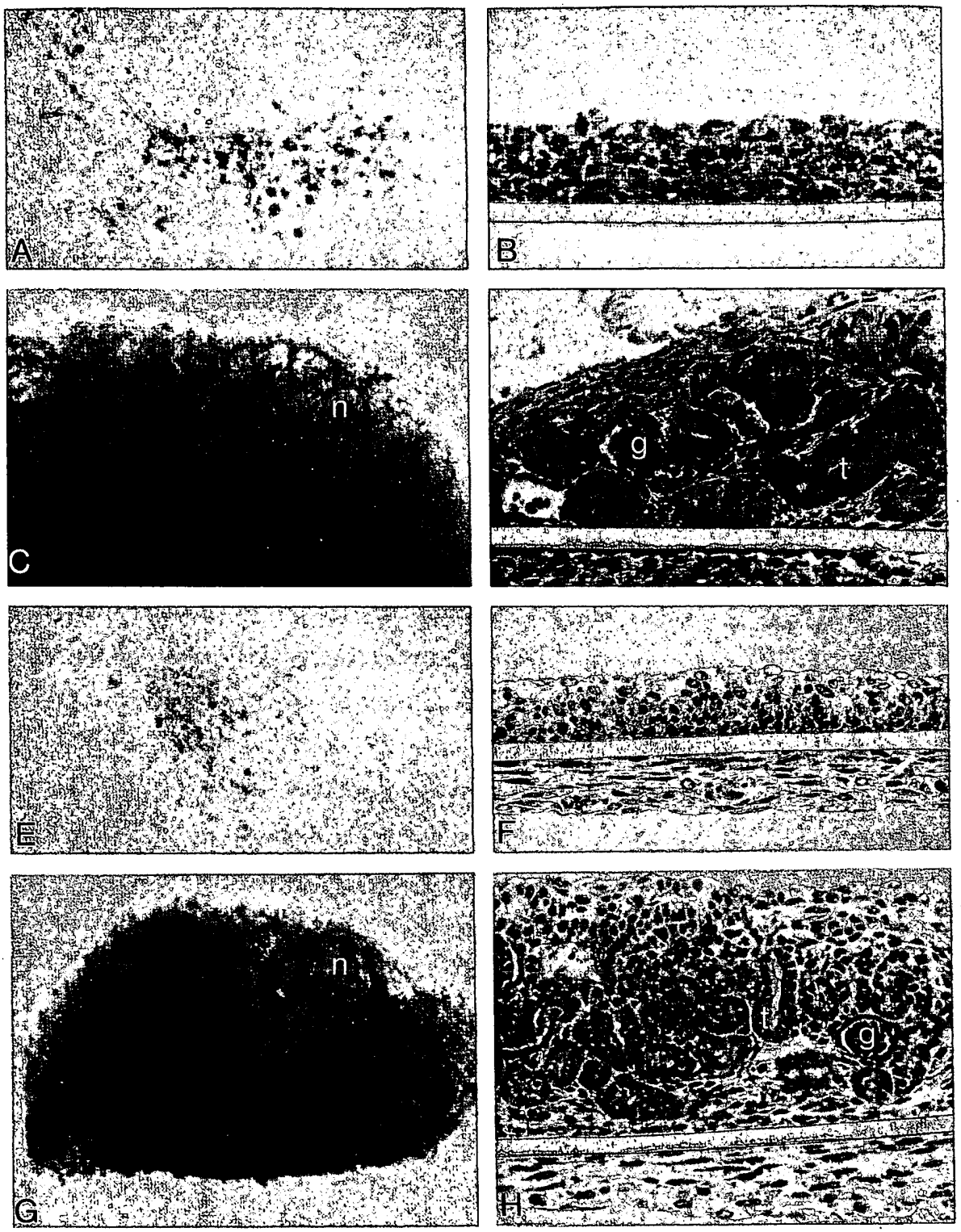

FIG. 1. Induction of nephron differentiation by embryonic spinal cord and *Wnt-1*-expressing cells. Whole mounts (A, C, E, G) and corresponding sections (B, D, F, H) of isolated metanephric blastemata cultured with the following: (A and B) no inducing tissue; (C and D) embryonic spinal cord; (E and F) NIH3T3 cells expressing a frameshift mutant *Wnt-1* allele; (G and H) NIH3T3 cells expressing wild-type *Wnt-1*. In each case, blastemata were cultured above a polycarbonate filter (visible in B, D, F, H) and the inducing tissue or cells cultured below. Blastemata cocultured with either embryonic spinal cord or *Wnt-1*-expressing cells differentiated into nephrons, while control cultures failed to differentiate. n, nephrons; g, glomeruli; t, tubules.

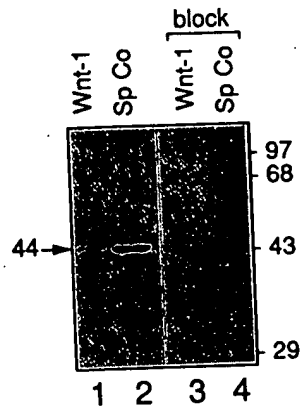

FIG. 2. Embryonic rat spinal cord expresses Wnt-1 protein. Immunoblotting using a monoclonal anti-Wnt peptide A antibody shows that Gestation Day 13 embryonic spinal cord (lane 2) expresses a 44-kDa form of Wnt-1 protein that comigrates with the species secreted from C57MG mammary epithelial cells transformed by *Wnt-1* (lane 1). Detection of these bands was inhibited by preblocking the antibody with the appropriate peptide (lanes 3 and 4). Relative molecular weights are marked in kilodaltons.

of Wnt-1 protein secreted by cultured cell lines expressing *Wnt-1* cDNA (Fig. 2).

Since Wnt proteins are not yet available in purified form, we used fibroblast cell lines expressing cloned *Wnt-1* cDNA as sources of Wnt-1 protein. Such cells undergo no obvious change in phenotype, but have previously been shown to secrete Wnt-1 protein and to induce paracrine transformation of neighboring *Wnt*-responsive cells in coculture experiments (Jue *et al.*, 1992). To test the potential of *Wnt-1* to induce nephron formation, blastemata were cultured with Wnt-1-producing fibroblasts or several control cell populations. No evidence of differentiation was observed in blastemata cultured with control fibroblasts (Figs. 1E and 1F). In contrast, blastemata cultured with Wnt-1-producing cells differentiated into nephrons, a result comparable to that observed with embryonic spinal cord (Fig. 1). Cell condensations were visible in the cultures within 24 hr of coculture, and sectioning after 5 days revealed extensive epithelial tubules and glomeruli (Figs. 1G and 1H). Similar effects were consistently observed using either Rat-2/*Wnt-1* or NIH3T3/*Wnt-1* cells as inducers, but no such differentiation was observed with any of the negative control cell populations (Table 1). Although it remains to be determined whether Wnt-1 protein alone is capable of inducing nephrogenesis, the inducing activity exhibited by fibroblasts in this system is dependent on their expression of Wnt-1.

Collectively, these results imply that the ability of embryonic spinal cord to act as an inducer of nephrogenesis may result from its production of Wnt-1 protein. This notion is further supported by the timing of *Wnt-1* expression in the neural tube (Shackleford *et al.*, 1987; Wilkinson *et al.*, 1987), which is coincident with the ability of embryonic spinal cord to act as an inducer of nephrogenesis (Grobstein, 1955). During embryogenesis, *Wnt-1* expression is detected exclusively in the developing central nervous system and it is therefore unlikely to mediate renal morphogenesis *in vivo* (Wilkinson *et al.*, 1987; Nusse and Varmus, 1992). However, there is growing evidence of functional similarity between certain *Wnt* genes, and *Wnt-5b* and *Wnt-7b* RNA have been detected in the kidney (Nusse and Varmus, 1992; Gavin *et al.*, 1990). Moreover, other members of the *Wnt* gene family may be transiently expressed in this tissue during development.

Renal epithelial morphogenesis is characterized by a series of distinct changes in cell behavior and gene expression. When metanephric blastema cells are induced by embryonic spinal cord, one of the earliest demonstrated responses is increased cell proliferation (Saxen, 1987). Such a response is consistent with the known mitogenic effects of *Wnt-1* in the mouse mammary gland and of *Drosophila wingless* in the developing Malpighian tubule (Nusse and Varmus, 1992; Skaer, 1989). After their initial proliferation, induced blastema cells begin to undergo mesenchymal to epithelial conversion (Saxen, 1987). This is accompanied by increased expression of the cell adhesion molecule E-cadherin, which can play a key role in generating the epithelial phenotype (Vestweber *et al.*, 1985). In this regard it is notable that *Wnt-1* has been shown to cause elevated expression of cadherins in *Wnt*-responsive cell lines and in some cases to induce a more epithelioid cell morphology (Bradley *et al.*, 1993; Hinck *et al.*, 1994). It is therefore possible that

TABLE 1
NEPHRON-INDUCING ACTIVITY OF CELLS EXPRESSING Wnt-1 PROTEIN

Cells or tissue	Cultures with nephrons/ cultures established
No inducer	0/5
Spinal cord	5/5
NIH3T3	0/9
NIH3T3/MV7	0/6
NIH3T3/*Wnt-1*	6/6
Rat-2	0/6
Rat-2/MV7	0/21
Rat-2/fs*Wnt-1*	0/6
Rat-2/*Wnt-1*	12/12

Note. Isolated metanephric blastemata were cocultured with embryonic spinal cord, uninfected NIH3T3, and Rat-2 fibroblasts or cell populations infected with the retroviral vector MV7 (expressing *neo* alone), MV7 derivatives expressing a frameshift (fs) mutant allele of *Wnt-1*, or wild-type *Wnt-1*. Cultures were scored for the presence of nephrons after 6 days. Cells expressing Wnt-1 induced nephron formation in every case examined.

the initial cellular responses leading to nephron formation may be attributable directly to Wnt protein signals.

In conclusion, the results presented here demonstrate that expression of *Wnt-1* in fibroblast cell lines confers the ability to induce renal epithelial morphogenesis *in vitro*, a property not attributable to any other known genes or factors. Expression of Wnt-1 may account for the inducing potential of embryonic spinal cord in this system and raises the possibility that a member of the *Wnt* gene family mediates nephron formation *in vivo*. The ability to recapitulate this differentiative process *in vitro* provides a powerful means to analyze the mechanisms by which mammalian *Wnt* signals mediate their developmental effects.

MATERIALS AND METHODS

Gestation Day 13 rat kidney rudiments were incubated in 0.2% collagenase for 15 min and metanephric blastemata separated from ureteric buds by microdissection. To verify that blastemata preparations were devoid of ureteric bud, 50,000 cells from trypsinized preparations were analyzed with a ureteric bud-specific marker, FITC dolichos biflorus (FITC-DB), by fluorescence-activated cell sorting. Less than 2% of cells of blastemata preparations bound FITC-DB, while 96% of the cells of isolated ureteric buds were FITC-DB positive. To generate cells expressing Wnt-1, NIH3T3 and Rat-2 fibroblasts were infected with the replication-defective retroviral vector MV*Wnt-1* (Jue *et al.*, 1992) and 30–100 infected colonies pooled after selection in G418. Control populations were infected with MVfs*Wnt-1*, which expresses a functionally defective frameshift Wnt-1 mutant, or with the parental vector MV7, which carries *neo* alone (Jue *et al.*, 1992). For transfilter coculture experiments, three microdissected metanephric blastemata were placed together on the upper surface of a 0.4-μm-poe polycarbonate filter. Where indicated, a fragment of Gestation Day 13 spinal cord was attached to the bottom filter surface with 1% agar. Twenty-four hours prior to the addition of blastemata, cell lines were plated on the lower filter surface at a density of 3×10^4 cells/cm^2. Transfilter cultures were grown at the air/medium interface in DMEM/10% FCS at 37°C in an atmosphere of 5% CO_2 for 6 days and scored for the presence of tubules by whole mount examination at 50× magnification. Cultures were then fixed and embedded in methacrylate and 2-μm-thick sections examined at 200× magnification to confirm whole mount observations.

For immunoblotting, spinal cord dissected from Gestation Day 13 rat embryos was solubilized directly in Laemmli buffer. Secreted Wnt-1 protein in the medium of C57MG cells infected with MV*Wnt-1* retrovirus was concentrated by sedimentation at 100,000*g* and solubilized in Laemmli buffer (Jue *et al.*, 1992). Wnt-1 protein was detected with a monoclonal antibody directed against Wnt-1 peptide A as described previously (Jue *et al.*, 1992) and visualized with secondary antibody conjugated to horseradish peroxidase followed by reaction with enhanced chemiluminescence substrates.

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Use of MMTV-*Wnt-1* transgenic mice for studying the genetic basis of breast cancer

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***Wnt-1* was first identified as a protooncogene activated by viral insertion in mouse mammary tumors. Transgenic expression of this gene using a mouse mammary tumor virus LTR enhancer causes extensive ductal hyperplasia early in life and mammary adenocarcinomas in approximately 50% of the female transgenic (TG) mice by 6 months of age. Metastasis to the lung and proximal lymph nodes is rare at the time tumors are detected but frequent after the removal of the primary neoplasm. The potent mitogenic effect mediated by *Wnt-1* expression does not require estrogen stimulation; tumors form after an increased latency in estrogen receptor α-null mice. Several genetic lesions, including inactivation of *p53* and over-expression of *Fgf-3*, collaborate with *Wnt-1* in leading to mammary tumors, but loss of *Sky* and inactivation of one allele of *Rb* do not affect the rate of tumor formation in *Wnt-1* TG mice.** *Oncogene* (2000) **19,** 1002-1009.

Keywords: Wnt-1; breast cancer; cancer models; mammary; transgenic; MMTV

Introduction

Infection of most strains of mice, such as C3H, with mouse mammary tumor virus (MMTV) leads to a high incidence of mammary tumors (reviewed by Nusse, 1991). The sites of insertions by MMTV proviruses have been extensively mined in order to identify genes that are deregulated to cause tumorigenesis. *Wnt-1* was the first protooncogene to be cloned following activation by viral insertion in mouse mammary tumors (Nusse and Varmus, 1982). (Its initial name, *int-1*, was subsequently changed to *Wnt-1* because of its homology to the Drosophila *Wingless (Wg)* gene (Nusse *et al.*, 1991)). Insertional activation of *Wnt-1* occurs in approximately 70% of C3H mice that are chronically infected with MMTV (Nusse and Varmus, 1982). Other candidate protooncogenes that are sometimes activated by MMTV proviral insertions include two additional members of the *Wnt* family, *Wnt-3* (Roelink *et al.*, 1990) and *Wnt-10b* (Lee *et al.*, 1995); three members of the fibroblast growth factor family, *Fgf-3/int-2* (Dickson *et al.*, 1984), *Fgf-4/hst* (Peters *et al.*, 1989), and *Fgf-8/AIGF* (MacArthur *et al*, 1995); *Notch-4/int-3* (Lee *et al.*, 1995); and *int-6* (Asano *et al.*, 1997), encoding a subunit of the translation initiation factor eIF3. Some of these genes, such as *Fgf-3* and *Wnt-10b*, have been validated as oncogenes by transgenic expression (Kwan *et al.*, 1992; Lane and Leder, 1997; Muller *et al.*, 1990).

The *Wnt-1* gene encodes a member of a large family of secreted proteins that are cysteine-rich, glycosylated, and poorly soluble (reviewed by Nusse and Varmus, 1992). Presently, at least 18 distinct Wnt family members have been identified in mammals. Having a propensity to associate with the extracellular matrix, Wnts act on both Wnt-producing and adjacent cells through cell surface receptors to control cell fate and patterning (reviewed by Nusse and Varmus, 1992). In mice, *Wnt-1* is expressed exclusively in the developing central nervous system (CNS) and adult testes (Jakobovits *et al.*, 1986; Shackleford and Varmus, 1987; Wilkinson *et al.*, 1987), and it is required for CNS patterning and development of the midbrain and cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Its Drosophila ortholog, *Wg*, controls segment polarity and many other developmental processes (Wodarz and Nusse, 1998).

A brief overview of the Wnt signaling pathway

One of the major intracellular responses to Wnt-1 signaling is to stabilize and increase the level of cytosolic β-catenin (Figure 1), a multi-functional protein that associates with membrane-bound E-cadherin, as well as several DNA binding proteins, such as members of the TCF/LEF family (reviewed by Kinzler and Vogelstein, 1996). Heterodimers of β-catenin and transcriptions factors translocate to the nucleus and transactivate a number of genes, including c-*myc* (He *et al.*, 1998), *cyclin D1* (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999), *WISPs* (Pennica *et al.*, 1998), and possibly *cyclooxygenase-2* (Howe *et al.*, 1999). Depending upon the cell type, Wnt signaling activates different genes, affecting various stages of development and several types of cancer.

The receptors for Wnts have been identified as a class of seven transmembrane proteins known as Frizzled (Fz) (Bhanot *et al.*, 1996). The ligand-receptor interaction is facilitated by extracellular proteoglycans and inhibited by Fz-related proteins, dickkopf, and cerberus. After binding a Wnt ligand, Fz transmits a signal to cytoplasmic phosphoproteins in the disheveled (Dvl) family via unknown mechanisms. Dvl inhibits the constitutively active kinase activity of glycogen synthase kinase type 3 (GSK3), which normally phosphorylates β-catenin and targets it for degradation. Cytosolic levels of β-catenin are additionally regulated by adenomatous polyosis coli (APC), which targets β-catenin for proteosome-mediated degradation, and by another large protein, Axin, also an inhibitor of Wnt signaling. A complex

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containing APC, Axin, β-catenin, GSK3, and GSK-binding protein/Frat-1 has been observed in lysates prepared from certain cell types (reviewed by Barish and Williams, 1999).

Several members of the Wnt family transform cultured cells, when overexpressed. For example, overexpression of *Wnt-1*, *Wnt-2*, *Wnt-3*, and *Wnt-3a* (but not *Wnt-4*, *Wnt-5a*, *Wnt-5b*, and *Wnt-7b*) leads to morphological transformation of mammary epithelial cells such as C57MG (Brown *et al*., 1986; Shimizu *et al*., 1997). Continued overexpression is required for the transformation phenotype induced by *Wnt-1* (Li *et al*., 1999; Mason *et al*., 1992).

Wnt-1 is not normally expressed in the mammary gland, nor has it been directly implicated in human breast cancer. However, several other *Wnt* family members are expressed in breast tissue, and some are overexpressed in breast tumors (reviewed by Bergstein and Brown, 1999). In addition, genes encoding several components and targets of the Wnt signaling pathway, including β-catenin, APC, E-cadherin, cyclin D1, c-*myc*, and WISPs, have been found to be mutated or deregulated in several types of human tumors, such as breast cancer (Bieche *et al*., 1999), colon cancer (He *et al*., 1998), melanoma (Rimm *et al*., 1999; Rubinfeld *et al*., 1997), hepatocellular carcinoma (de La Coste *et al*., 1998), and pilomatricoma (Chan *et al*., 1999).

The Wnt-1 *transgenic (TG) mouse model*

Wnt-1 TG mice were initially made to test the oncogenicity of *Wnt-1* (Tsukamoto *et al*., 1988). The transgene (Figure 2), is controlled by the *Wnt-1* promoter and an MMTV LTR inserted upstream of the gene in the opposite transcriptional orientation, in a fashion reminiscent of a typical viral insertion into the *Wnt-1* locus in MMTV-induced tumors. Ectopic *Wnt-1* expression exerts a potent mitogenic effect on mammary epithelium; ductal hyperplasia is noticeable in the mammary end-buds by 18 days of gestation (Cunha and Hom, 1996) and very apparent 2 weeks after birth in the TG females (Lin *et al*., 1992). Because of the extensive ductal hyperplasia, female TG mice can not deliver milk to their young.

About 50% of virgin female *Wnt-1* TG mice in the SJL strain develop adenocarcinomas by 6 months of age; the rest succumb to tumors by 1 year. Breeding females develop tumors slightly earlier than virgin mice (Shackleford *et al*., 1993; Tsukamoto *et al*., 1988). This acceleration may be caused by either hormonal influence on cell growth or the increased mass of the mammary epithelium attributed to pregnancies and lactation. Hyperplasia is also extensive in the primary mammary glands of adult male TG mice; about 15% of them develop palpable mammary tumors by 1 year of age (Kwan *et al*., 1992; Tsukamoto *et al*., 1988). Although metastasis does not seem to occur frequently at the time mammary tumors are detected (Tsukamoto *et al*., 1988), the majority of female *Wnt-1* TG mice develop lymph node and/or lung metastasis after removal of the primary tumor (L Godley and WP Hively, unpublished observation).

Tumors found in Wnt-1 TG mice are usually moderately differentiated and comprised of ducts with multiple layers of epithelial cells, that show significantly higher than normal nucleus-to-cytoplasm ratio and occasionally pleomorphic nuclei and mitotic figures. The lumens usually contain pyknotic cells suggestive of apoptosis. Widespread necrosis and hemorrhage are sometimes noticeable in these tumors. In addition, extensive fibrosis is present in neoplasms induced by the *Wnt-1* transgene. Hyperplastic glands of *Wnt-1* TG mice also display a prominent fibrotic response, which may start as early as 7 days postnatally in TG females (G Cunha, personal communication).

Variations in genetic backgrounds usually do not influence the time course of tumor development mediated by the *Wnt-1* transgene (Bocchinfuso *et al*., 1999; Donehower *et al*., 1995; Shackleford *et al*., 1993; Tsukamoto *et al*., 1988). The original TG line was made in C57BL/6 X SJL F1 mice. Subsequently, interbreedings with other strains (FVB/N, BALB/c, 129/J, C58BL/6) have been found to be similar to SLJ in tumor latency (Table 1). But a much longer latency has been observed in some mixed backgrounds (C Alexander, personal communication).

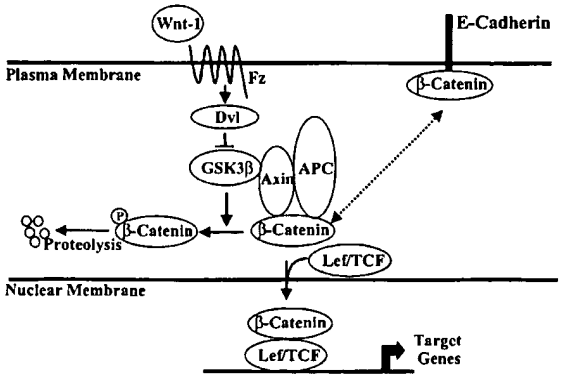

Figure 1 Illustration of the Wnt signal transduction pathway (kindly provided by J-M Li). See text for explanation

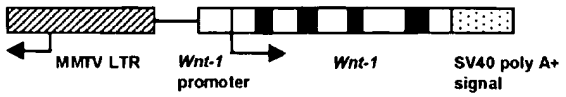

Figure 2 *Wnt-1* transgene construct. The 7 kb transgene contains the MMTV-LTR approximately 1 kb upstream of the mouse *Wnt-1* gene. The MMTV-LTR was placed in the opposite transcriptional orientation and is used as an enhancer. The *Wnt-1* coding sequences are shown as filled boxes. A fragment containing the SV-40 splice and polyadenylation sites (850 bp) was placed downstream of the last exon of *Wnt-1*

Table 1 Genetic lesions crossed to the MMTV-*Wnt-1* transgene

Genotype	*Genetic background*	*References*
MMTV-*Fgf-3* TG	FVB/N	Muller *et al*., 1990; Kwan *et al*., 1992
Sky −/−	129/Sv × C57BL/6	Lu *et al*., 1999; WP Hively (unpublished)
p53 −/−	129/Sv	Donehower *et al*., 1992, 1995
ERα −/−	C57BL/6	Lubahn *et al*., 1993; Bocchinfuso *et al*., 1999
MMTV-TGF	C57BL/6	A Chytil, Y-L Chen and HL Moses (unpublished)

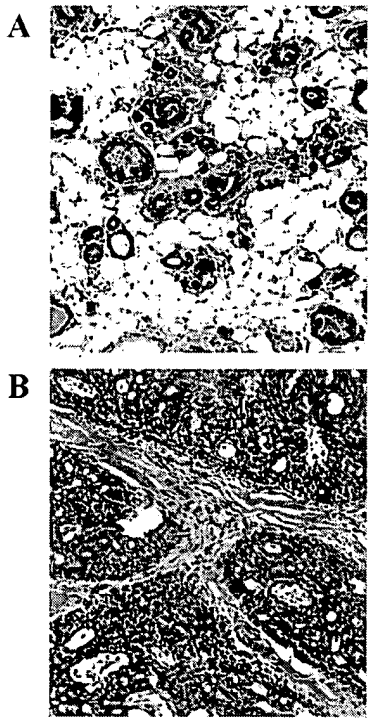

Figure 3 Histology of mammary glands from an 18-week-old MMTV-*Wnt-1* TG virgin female. (**a**) Hyperplastic mammary gland (25×). (**b**) Mammary adenocarcinoma (25×)

Many genetic lesions and epigenetic changes, such as levels of mammogenic hormones, have been implicated in breast carcinogenesis. However, the complex molecular interplay leading to breast cancer is very poorly understood. All mammary epithelial cells expressing the transgene in the *Wnt-1* TG line are at risk for tumor development. Indeed, ductal hyperplasia occurs throughout the mammary tissue early in development, yet tumors appear stochastically after several months. Therefore, other cooperative events must have accompanied expression of the *Wnt-1* transgene in the few cells that expanded into tumors. A number of methods have been applied to uncover these synergistic events. Among them are hormonal manipulations, insertional activation of protooncogenes using retroviral infection, and breeding with TG and knockout mice carrying other genetic lesions implicated in breast cancer.

Hormonal and stromal influences on Wnt-1*-induced hyperplasia and tumors*

Estrogen is essential in mammary development and plays a very important role in carcinogenesis of the breast (reviewed by Pike *et al*., 1993). It stimulates ductal morphogenesis and branching through nuclear receptors–ERα and possibly the recently identified ERβ (Kuiper *et al*., 1996; Mosselman *et al*., 1996). *ERα* is expressed in both mammary epithelium and the stroma (Daniel *et al*., 1987). *ERβ* is detectable in mammary tissues at low levels, but its role in mammary proliferation remains elusive (Krege *et al*., 1998).

Hyperplastic ductal growth in the *Wnt-1* TG animals persists despite estradiol deprivation due to ovariectomy (Bocchinfuso *et al*., 1999; Lin *et al*., 1992). Albeit delayed, tumors continue to form in ovariectomized mice (Bocchinfuso *et al*., 1999), suggesting that *Wnt-1* does not require estrogen signaling for stimulating proliferation and inducing tumors. These results were confirmed and strengthened by experiments in which the MMTV-*Wnt-1* transgene was crossed into the *ERα* knockout (*ERKO*) mice. In homozygous *ERKO* mice, mammary glands are underdeveloped, with rudimentary ducts confined to the nipple area (Lubahn *et al*., 1993). The presence of the *Wnt-1* transgene stimulated hyperplastic ductal growth in *ERKO* mice, and the females developed mammary tumors at twice the age of *Wnt-1* TG females with one or two intact *ERα* genes (Bocchinfuso *et al*., 1999). It remains to be determined if the increased latency to tumor development observed in ovariectomized or *ERKO* mice is the result of reduced mass of mammary epithelium, the loss of cooperative functions of ER signaling in *Wnt-1*-induced oncogenesis, or both.

The majority of human breast tumors are ERα-positive and respond to anti-hormone therapy; however, most malignant tumors are ERα-negative (McGuire and Clark, 1985). Together with the fact that only a small percentage of mammary epithelial cells express *ERα* (Petersen *et al*., 1987; Ricketts *et al*., 1991), it has been suggested that breast cancer may initiate from ER-positive cells but become ER-negative and estrogen-independent in its growth at later stages (Moolgavkar *et al*., 1980). The observation that mammary tumors arise in both *ERKO* and ovariectomized mice supports an alternative model that a fraction of breast cancers may directly evolve from ERα-negative cells (Nandi *et al*., 1995), an idea that needs to be tested with other oncogenic transgenes.

The potent mitogenic effect of Wnt-1 on mammary epithelial cells may not depend upon other mammogenic hormones either. For example, a similar degree of abnormal side branching was observed in mammary epithelial transplants derived from *Wnt-1* TG mice that were either wild-type or nullizygous for progesterone receptor α (*PRα*, C Brisken and R Weinberg, personal communication). Likewise, in ovariectomized and/or adrenalectomized mice, Wnt-1 continued to stimulate hyperplastic growth in transplanted and reconstituted glands (Edwards *et al*., 1992; Lin *et al*., 1992).

The reciprocal interactions between parenchyma and stroma are important in mammary development, remodeling, and carcinogenesis (reviewed by Cunha and Hom, 1996). For example, signaling through the epidermal growth factor receptor (EGFR) in the mesenchyme is required for ductal growth and branching morphogenesis, since epithelium transplanted from wild-type mice fails to proliferate in the fat-pad from *EGFR*-null mice (Wiesen *et al*., 1999). Interestingly, this requirement also seems to be diminished in *Wnt-1* TG mice. Transplantation of epithelium from *Wnt-1* TG animals into the fat-pad of *EGFR* nullizygous mice only modestly impaired hyperplastic growth (G Cunha, personal communication). Epithelial-stromal interactions in tumor formation have also been studied by experiments in which mammary epithelial cells from *Wnt-1* TG animals were transplanted into rat mammary fat-pad. Transplanta-

tion led to fibrotic proliferation in rat mesenchyme (G Cunha, personal communication), suggesting that the alteration of stromal differentiation is mediated by the *Wnt-1*-expressing epithelial cells. Wnt-mediated epithelial-mesenchymal interactions have also been reported in other tissues. For example, Wnt-induced mesenchymal reactions may regulate axonal growth and guidance in developing limbs. Several members of the *Wnt* family expressed in limb ectoderm induce production of neurotrophin-3 in the underlying mesenchyme (Patapoutian *et al*., 1999).

Collaboration between Wnt-1 *and other genes in oncogenesis*

Mammary tumors induced by MMTV occasionally show transcriptional activation of both *Wnt-1* and *fibroblast growth factor3* (*Fgf3*, Peters *et al*., 1986), suggesting that these two genes collaborate in oncogenesis. Fgf3 belongs to a family of heparin-binding proteins that are both mitogenic and angiogenic. Signaling by FGFs is mediated by transmembrane receptors (FGFRs) that phosphorylate and activate several substrates, leading to the activation of mitogen-activated protein kinases (reviewed by Faham *et al*., 1998). Although Wnts and FGFs act through very different pathways, they are both required for development of primary body axis, neural axis, limbs, and other structures, suggesting that these two families of growth factors may collaborate in development in ways that resemble synergistic roles in tumor formation.

Transgenic female mice expressing MMTV-*Fgf3* show extensive mammary hyperplasia but rarely develop tumors (Muller *et al*., 1990). When this transgene was bred into *Wnt-1* TG mice, tumors developed faster in bi-transgenic females than in females bearing either transgene alone, providing direct evidence of cooperation between these two growth factors (Kwan *et al*., 1992). The acceleration is even more dramatic in the bi-transgenic males. Additional evidence of synergistic interactions between *Wnt-1* and members of the *Fgf* family comes from infection of *Wnt-1* TG animals with MMTV (Shackleford *et al*., 1993). Infection accelerates tumor formation, and up to ten tumors per mouse were observed in infected animals. Approximately 40% of the mammary tumors showed insertional activation of *Fgf3*, a small percentage of them had insertional activation of both *Fgf3* and *Fgf-4* or *Fgf4* alone. Another member of the *Fgf* family, *Fgf-8*, was also found to be insertionally-activated and/or overexpressed in some of these tumors (Kapoun and Shackleford, 1997; MacArthur *et al*., 1995). Collaboration between members of the Wnt and FGF families has also been observed in experiments in which infection of MMTV-*Fgf3* TG mice with MMTV led to frequent viral insertions in *Wnt-1* or *Wnt-10b* loci (Lee *et al*., 1995).

Tumor growth factor β (TGFβ) stimulates cell growth under some conditions, but, more commonly, inhibits cell proliferation, especially in the mammary gland (reviewed by Massague, 1998). For example, transgenic expression of *TGFβ* inhibits tumor formation in mice expressing an MMTV-*TGFα* transgene (Pierce *et al*., 1995). But in a recent cross between our MMTV-*Wnt-1* TG mice and MMTV-*TGFβ* TG animals, no effects were observed on the rate of tumor appearance, histology, or the size of the tumor induced by the *Wnt-1* transgene (A Chytil, Y-L Chen and HL Moses, personal communication). Assuming adequate levels of expression, it appears TGFβ cannot inhibit proliferation of mammary epithelia stimulated by the *Wnt-1* transgene.

Collaboration between Wnt-1 *and loss of a tumor suppressor gene*

Several tumor suppressor genes are mutated or downregulated in human breast cancer. Inherited mutations of some of them predispose to breast neoplasm. For example, mutations of *BRCA-1* and *BRCA-2* are found in approximately 50% and 30%, respectively, of families predisposed to breast cancer (Ford *et al*., 1998). Somatic *p53* mutations are found in about 35% of sporadic and 85% of familial breast cancers (Crook *et al*., 1998), and germline alterations of *p53* are associated with a predisposition to several cancers, including breast cancer (the Li-Fraumeni syndrome). *RB*, a cell cycle regulator, is also mutated in a small percentage of sporadic human breast cancers (Berns *et al*., 1995). In addition, p21/WAF1/CIP1, a cyclin-dependent kinase inhibitor that regulates G1-S cell cycle progression, is downregulated in some breast tumors, especially those with poor prognosis (Jiang *et al*., 1997; Wakasugi *et al*., 1997).

The impact of the loss of a tumor suppressor gene on tumorigenesis has been documented in animal models using targeted gene disruption, loss of heterozygosity (LOH) assays, and transgenic overexpression of a dominant-negative version of a tumor suppressor gene. Mice deficient for the *p53* tumor suppressor gene (*p53*+/− and *p53*−/−) develop tumors of non-epithelial origin (Donehower *et al*., 1992). To analyse the effect of *p53* inactivation on mammary oncogenesis, *p53* knockout mice were bred with *Wnt-1* TG mice (Donehower *et al*., 1995). *p53* nullizygotes (both females and males) expressing the *Wnt-1* transgene develop mammary tumors much earlier than mice containing at least one wild-type allele, suggesting that inactivation of *p53* plays an important role and collaborates with *Wnt-1* in mammary oncogenesis. In addition, *p53*-null tumors are more anaplastic and less fibrotic than tumors that carry at least one copy of the *p53* gene (Donehower *et al*., 1995).

Although the absence of one copy of *p53* did not significantly alter the time at which MMTV-*Wnt-1* transgene induced tumors appeared, approximately 50% of the tumors in *p53*-heterozygous, *Wnt-1* TG mice displayed loss of the wild-type locus. This frequent occurrence of LOH contrasts with the very rare loss of the wild-type *p53* allele in mammary tumors from *p53* heterozygotes carrying an MMTV-c-*myc* transgene (Elson *et al*., 1995; McCormack *et al*., 1998). It is notable that inactivation of *p53* collaborates with MMTV-c-*myc*, MMTV-H-*ras*, and MMTV-*neu* transgenes to produce lymphomas and salivary tumors, but rarely mammary tumors (C-X Deng, personal communication, Elson *et al*., 1995; Hundley *et al*., 1997).

Mutations in *BRCA-1* and *BRCA-2* are often associated with loss of *p53* in breast carcinogenesis in humans (Crook *et al*., 1998). Induction of mammary

tumors in the mouse by mammary-specific *Brca1* inactivation is dramatically accelerated by inactivation of *p53* (Xu *et al*., 1999). However, loss of one allele of *Brca-1* (T Wynshaw-Boris, personal communication) or *Brca-2* (XS Cui and LA Donehower, personal communication) does not seem to influence the kinetics of tumor formation induced by the *Wnt-1* transgene. It remains to be determined whether tumors that are heterozygous for *Brca1* or *Brca2* show LOH or alteration in karyotype. The availability of mice that carry loxP-flanked (floxed) alleles of *Brca1* and *Brca2* will permit better tests for synergy between the loss of *Brca1* or *Brca2* and inheritance of a *Wnt-1* transgene in tumor formation.

Germline mutations in one copy of the *Rb* gene predispose humans to retinoblastomas and osteosarcomas. Mice nullizygous for *Rb* die during embryogenesis; heterozygotes develop tumors primarily in the pituitary and thyroid glands but rarely in mammary glands (Jacks *et al*., 1992). To determine if the loss of *Rb* affects the development of tumors in *Wnt-1* TG animals, we have crossed *Wnt-1* TG animals with mice heterozygous for *Rb*. Absence of one allele of *Rb* did not affect the age at which the tumor was detected, and none of 25 tumors examined by restriction mapping showed loss of the wild-type locus (WP Hively, unpublished). The lack of acceleration may be due to the complementary expression of one or both of the other two members of the *Rb* gene family (*p107* and *p130*) in the mouse mammary gland. In fact, the presence of normal *p107* alleles has been shown to inhibit *Rb* deficiency-mediated tumor formation in the mouse retina (Robanus-Maandag *et al*., 1998). Elimination of all three members of the *Rb* gene family in *Wnt-1* TG mice would further clarify the role of their inactivation in mammary oncogenesis. One way to eliminate their functions is to generate transgenic mice expressing the gene encoding the amino terminal domain (T_{121}) of the Simian virus 40 T antigen, which inactivates all three members of the Rb family (Saenz Robles *et al*., 1994; Symonds *et al*., 1994).

Inactivation of one or both alleles of *p21* did not accelerate tumor formation in *Wnt-1* TG mice (Jones *et al*., 1999). But, interestingly, tumors from *p21*+/− mice grew significantly faster, with a higher mitotic index and increased cyclin D1-associated phosphorylation of Rb, than those from either *p21*+/+ or *p21*−/− mice (Jones *et al*., 1999).

Additional tumor suppressor genes that collaborate with the *Wnt-1* transgene to induce tumor formation may be identified by scanning the whole genome for LOH. Application of this approach has led to the identification of two regions on mouse chromosomes 9 and 16 that are frequently deleted in insulinomas and carcinoid tumors in TG mice expressing the Simian virus 40 *large T antigen* (Dietrich *et al*., 1994). Furthermore, using this technology in F1 hybrid mice between FVB/N and *Mus musculus castaneus*, Radany and colleagues (1997) have found that a marker on chromosome 4 from *Mus musculus castaneus* was frequently lost in MMTV-*H-Ras* transgene-induced mammary tumors. In contrast, no single chromosome was preferentially lost in tumors occurring in F1 progeny of a similar cross between Wnt-1 TG SJL mice and *Mus musculus castaneus* (unpublished data of K Hong *et al*., cited in Radany *et al*., 1997).

Molecular characterization of tumors from Wnt-1 TG animals

Chromosomal rearrangements including aneuploidy, chromosomal translocations and duplications, and amplification of selected genes are common in tumor cells (reviewed by Wright, 1999). Loss of *p53* function frequently leads to deregulated cell cycle control and chromosomal instability, which favors tumor growth (reviewed by Prives and Hall, 1999). Mammary tumors from *Wnt-1* TG mice with one or two functional copies of *p53* display occasional chromosomal abnormalities as shown by comparative genome hybridization (CGH, Kallioniemi *et al*., 1992), which detects regions of expansion and deletion in all chromosomes. As expected, *Wnt-1* induced tumors without any *p53* function usually have more than one chromosomal abnormality. Tumors that arose in *p53* heterozygotes and experienced LOH at the *p53* locus displayed even more extensive alterations (at least three regions of DNA gain or loss) (Donehower *et al*., 1995).

In general, it is difficult to anticipate what specific genes in an amplified chromosomal region may have synergized with an oncogenic transgene to induce neoplasm. But the distal region of chromosome 7, which was amplified in a *Wnt-1*-induced *p53*−/− tumor, is the site of *Fgf3* (Donehower *et al*., 1995). Molecular hybridization using an *Fgf3*-specific probe confirmed that *Ffg3* was amplified and abundantly expressed in this tumor (Donehower *et al*., 1995). This is different from human breast cancer, in which the syntenic region of chromosome 8q is frequently amplified (Brison, 1993; Lammie *et al*., 1991; Theillet *et al*., 1989), but *Fgf3* mRNA is not detected (Penault-Llorca *et al*., 1995); however, a linked gene, *PRAD-1*/*CycinD1*, is usually overexpressed in such tumors (Motokura *et al*., 1991).

Spectral karyotyping (SKY) labels each chromosome with a different color, allowing detection of chromosomal translocations and duplications (Liyanage *et al*., 1996). We have analysed some tumors from *Wnt-1* TG mice that were *p53*+/− or *p53*−/−. Translocations, trisomy, and aneuploidy have been detected in cells cultured from some of these tumors (Z Weaver and WP Hively, unpublished). Karyotype instability in mammary tumors has been reported in mammary-specific *Brca1* knockout mice (Xu *et al*., 1999) and other transgenic models including MMTV-c-*myc* (McCormack *et al*., 1998; Weaver *et al*., 1999).

As a physiologic response to genotoxins, *p53* is rapidly induced to cause cell cycle arrest and/or apoptosis. Inactivation of *p53* is often accompanied by accelerated cell growth and attenuated apoptosis (reviewed by Ko and Prives, 1996). *p53* deficiency (*p53*+/−, *p53*−/−) enhances cell proliferation in the *Wnt-1* transgene-derived tumors, but the modestly ongoing apoptosis that accompanies *Wnt-1* overexpression does not seem to be attenuated (Jones *et al*., 1997). Similarly, absence of one allele of *p53* does not affect the apoptotic index in mammary tumors induced by an MMTV-c-*myc* transgene (McCormack *et al*., 1998).

Normal telomeres are essential to cell survival. Telomerase is usually activated in human cancer cells, presumably to overcome shortened telomeres due to excessive cell replication (reviewed by de Lange and DePinho, 1999). Normal telomeres (20–50 kb) are

present in both hyperplastic glands and carcinomas from *Wnt-1* TG mice, regardless of the *p53* status (Broccoli *et al*., 1996). Interestingly, despite the presence of long telomeres, telomerase activity and the RNA component of the enzyme were consistently upregulated in these tumors compared with normal and hyperplastic glands (Broccoli *et al*., 1996), suggesting that activation of the telomerase machinery in at least some mammary tumors does not depend upon telomeric shortenings. Breeding *Wnt-1* TG animals with mice that carry a mutated gene for telomerase or a component of the telomeric complex (Blasco *et al*., 1997; Rudolph *et al*., 1999) will help address whether the telomere activation is required for mammary oncogenesis induced by the *Wnt-1* transgene.

Another approach to uncovering the molecular basis of tumorigenesis is to identify differentially-expressed genes during various stages of tumor formation. Several methods including subtractive hybridization, differential display, serial analysis of gene expression (SAGE), and cDNA expression array technology have been used. A number of genes have been found to be deregulated in *Wnt-1*-induced tumors and *Wnt-1*-transformed cells by these and other methods. For example, using PCR to screen for differentially expressed tyrosine kinases, we found that *Sky*, which encodes a member of the Axl/Ufo family of receptor tyrosine kinases, is barely detectable in the mammary glands from virgin animals and in preneoplastic mammary glands, but is abundantly expressed in the mammary tumors of *Wnt-1* TG mice (Taylor *et al*., 1995). Recently, using a modified subtractive hybridization approach, Pennica *et al*. (1998) have found that two novel genes, *WISP1* and *2*, are overexpressed in *Wnt-1*-transformed mammary cells and that they are transcriptionally regulated by *Wnt-1* expression and aberrantly expressed in colon cancer.

Screening for upregulated genes in tumors might also help identify collaborating factors in tumor formation. But alteration of the transcriptional apparatus during neoplastic conversion may deregulate many non-collaborating genes. An example of such a non-synergistic element is *Sky*. Tumor development in *Wnt-1* transgenic mice was not affected by breeding *Sky* knockout mice (Lu *et al*., 1999) with *Wnt-1* TG animals (WP Hively, unpublished), suggesting that overexpression of *Sky* is not necessary for *Wnt-1* mediated oncogenesis.

Involvement of other components of the Wnt signaling pathway in mammary carcinogenesis

Many other components of the Wnt signaling pathway have been implicated in mammary tumorigenesis. Mutations in *APC*, a negative regulator of the Wnt signaling pathway, have been reported to confer an increased risk for development of breast cancer in Ashkenzi Jews (Redston *et al*., 1998; Woodage *et al*., 1998). *Min* mice, which carry a nonsense mutation at one *APC* locus, also have increased risk for mammary carcinomas after carcinogen treatment (Moser *et al*., 1993, 1995). With the use of additional TG mice, it will be interesting to determine if deregulated expression of other components of the Wnt-1 signaling pathway, such as over-expression of *β-catenin* and inactivation of *E-cadherin*, also induce mammary tumors.

All three members of the *Dvl* family (*Dvl1*, *Dvl2* and *Dvl3*) are expressed in mammary glands, with *Dvl1* being most abundant (Tsang *et al*., 1996). Dvl proteins transmit signals from the Fz receptor for Wnt-1 to β-catenin via unknown mechanisms (see Introduction). Mice nullizygous for *Dvl1* are normal except for abnormalities in social behavior and sensorimotor gating (Lijam *et al*., 1997). The mammary epithelia lacking the dominant member of this family might be expected to respond poorly to *Wnt-1* induced cell proliferation and tumor formation. But *Dvl1* nullizygosity did not affect the rate of tumor formation in *Wnt-1* TG mice (N Lijam, WP Hively, HE Varmus and T Wynshaw-Boris, unpublished). Since *Dvl2* and *Dvl3* are also expressed in the mammary gland, they might have substituted for *Dvl1* in mediating the Wnt-1 signal.

Syndecan-1 is a member of the transmembrane proteoglycan family that regulates cell morphology and growth (Leppa *et al*., 1992). Proteoglycans facilitate the binding of Wnt ligands to Fz receptors (Lin and Perrimon, 1999; Wodarz and Nusse, 1998). Consistent with this finding, *Wnt-1* TG mice that carry two null alleles of *syndecan-1* very rarely develop tumors (C Alexander, personal communication), suggesting that syndecan-1 may be an important factor in mediating Wnt-1 signaling in the mammary gland.

Prospects

Although initially developed to document the oncogenic potential of *Wnt-1*, our line of MMTV-*Wnt-1* TG mice has been useful in studying many aspects of mammary tumorigenesis: the cooperation between cancer genes, the influence of estrogen receptors and growth hormones, and the concomitant changes in genomic instability and gene expression.

Different stages of tumor progression can be discerned in *Wnt-1* TG mice, and some of the collaborative lesions accompanying *Wnt-1* overexpression in tumor formation have been defined. Therefore, this line may be a convenient source of hyperplastic glands and invasive and metastatic tumors for various approaches designed to identify molecular signatures of tumor progression. Comparing the expression profile of the *Wnt-1*-derived tumors with those of tumors derived from *Wnt-1* TG mice crossed with other genetically modified lines may offer additional insights into the complex nature of mammary oncogenesis. Additional benefits in the characterization of these tumors include identification of transcriptional targets of the Wnt-1 signaling pathway.

Recently, a novel method has been used to transduce oncogenes into somatic cells of a specific tissue (reviewed by Fisher *et al*., 1999) using sub-group A avian leukosis virus (ALV-A) as a vector. Transgenic expression of *tv-a*, encoding the receptor for ALV-A, from a cell type-specific promoter, permits tissue-specific infection with ALV-A, which does not produce infectious virus in mammalian hosts. Consequently, combinatorial effects of genetic lesions can be examined in a single TG line by infecting with mixtures of ALV-A viruses expressing different oncogenes. In addition, ALV-A expressing the gene encoding Cre

recombinase can be used to inactivate tumor suppressor genes flanked with loxP recombination sites. Since ALV infection requires mitotic cells which are widely available only during late pregnancy, breeding the *Wnt-1* transgene into mice expressing *tv-a* from a mammary-specific promoter may provide both replicating epithelial cells (eliminating the requirement for pregnancies) and a cancer predisposing factor allowing more rapid formation of tumors. The TVA technology may help test candidate collaborative events in context of the *Wnt-1* transgene.

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The Wnt family of cell signalling molecules in postimplantation development of the mouse.

McMahon AP, Gavin BJ, Parr B, Bradley A, McMahon JA.

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The mammalian Wnt gene family consists of at least ten members, all of which share a common structure. The N-terminus encodes a putative signal peptide sequence, suggesting that Wnt proteins are secreted. A number of absolutely conserved cysteine residues imply that inter- or intramolecular disulphide bonding is important to Wnt protein function. Wnt RNAs are localized to discrete regions of the postimplantation embryo and fetus, particularly within the developing central nervous system. Studies on Wnt gene expression strongly suggest that Wnt-mediated signalling is likely to be an important aspect of mouse development. One member of the family, Wnt-1, has been studied in some detail. By generating mutant alleles, we have demonstrated that Wnt-1 regulates regional development of the central nervous system at early somite stages. There is circumstantial evidence that some aspects of the pathway through which Wnt-1 action is mediated may be evolutionarily conserved. We propose that the Wnt family plays a major role in cell-cell interactions in the mouse.

Publication Types:
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The role of Wnt genes in vertebrate development.

Dickinson ME, McMahon AP.

Roche Institute of Molecular Biology, Department of Cell and Developmental Biology, Nutley, New Jersey 07110.

Over the past decade, many potential candidates for molecules involved in pattern formation in the vertebrate embryo have been identified. Manipulation of the expression of some of these factors has generated fascinating results that have allowed investigators to address their roles in embryogenesis. One such family consists of a group of putative cell signaling molecules related to the proto-oncogene Wnt-1. An accumulating body of evidence suggests that the Wnt-family plays a major role in several aspects of vertebrate development.

Publication Types:
- Review
- Review, Academic

PMID: 1388080 [PubMed - indexed for MEDLINE]

Control of β-Catenin Signaling in Tumor Development

JÜRGEN BEHRENS[a]

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ABSTRACT: **The wnt signal transduction pathway is involved in various differentiation events during embryonic development and leads to tumor formation when aberrantly activated. The wnt signal is transmitted to the nucleus by the cytoplasmic component β-catenin: in the absence of wnts, β-catenin is constitutively degraded in proteasomes, whereas in the presence of wnts β-catenin is stabilized and can associate with HMG box transcription factors of the LEF/TCF family. The LEF/TCF/β-catenin complexes activate specific wnt target genes. In tumors, β-catenin degradation is blocked by mutations of β-catenin or of the tumor suppressor gene product APC. As a consequence, β-catenin is stabilized, constitutive complexes with LEF/TCF factors are formed, and oncogenic target genes, such as *c-myc*, cyclin D1, and *c-jun*, are activated. Thus, control of β-catenin is a major regulatory event in normal wnt signaling and during tumor formation. It has been found that a multiprotein complex assembled by the cytoplasmic component conductin induces degradation of cytoplasmic β-catenin. The complex includes APC, the serine/threonine kinase GSK3β, and β-catenin, which bind to conductin at distinct domains. In colon carcinoma cells, forced expression of conductin downregulates β-catenin, whereas in normal cells mutants of conductin that are deficient in complex formation stabilize β-catenin. Fragments of APC that contain a conductin-binding domain also block β-catenin degradation. In *Xenopus* embryos, conductin inhibits the wnt pathway. *In situ* hybridization analysis shows that conductin is expressed in various embryonal tissues known to be regulated by wnts, such as the developing brain, mesenchyme below the epidermis, lung mesenchyme, and kidney. It is suggested that conductin controls wnt signaling by assembling the essential components of the β-catenin degradation pathway. Alterations of conductin function may lead to tumor formation.**

β-CATENIN: A PROTEIN WITH MULTIPLE FUNCTIONS

In mammals, β-catenin was originally identified as a component associated with cadherins, which are Ca^{2+}-dependent cell–cell adhesion molecules. β-Catenin and its relative plakoglobin (also termed γ-catenin) directly interact with the cytoplasmic tail of cadherins, but form mutually exclusive complexes. Via their amino-terminal domains, β-catenin and plakoglobin associate with the vinculin-related protein α-catenin, which in turn makes contact to actin filaments.[1] Catenins thus serve as a link between cadherins and the actin cytoskeleton (FIG. 1). Analysis of cytoplasmic de-

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ABSTRACT: **The wnt signal transduction pathway is involved in various differentiation events during embryonic development and leads to tumor formation when aberrantly activated. The wnt signal is transmitted to the nucleus by the cytoplasmic component β-catenin: in the absence of wnts, β-catenin is constitutively degraded in proteasomes, whereas in the presence of wnts β-catenin is stabilized and can associate with HMG box transcription factors of the LEF/TCF family. The LEF/TCF/β-catenin complexes activate specific wnt target genes. In tumors, β-catenin degradation is blocked by mutations of β-catenin or of the tumor suppressor gene product APC. As a consequence, β-catenin is stabilized, constitutive complexes with LEF/TCF factors are formed, and oncogenic target genes, such as *c-myc*, cyclin D1, and *c-jun*, are activated. Thus, control of β-catenin is a major regulatory event in normal wnt signaling and during tumor formation. It has been found that a multiprotein complex assembled by the cytoplasmic component conductin induces degradation of cytoplasmic β-catenin. The complex includes APC, the serine/threonine kinase GSK3β, and β-catenin, which bind to conductin at distinct domains. In colon carcinoma cells, forced expression of conductin downregulates β-catenin, whereas in normal cells mutants of conductin that are deficient in complex formation stabilize β-catenin. Fragments of APC that contain a conductin-binding domain also block β-catenin degradation. In *Xenopus* embryos, conductin inhibits the wnt pathway. *In situ* hybridization analysis shows that conductin is expressed in various embryonal tissues known to be regulated by wnts, such as the developing brain, mesenchyme below the epidermis, lung mesenchyme, and kidney. It is suggested that conductin controls wnt signaling by assembling the essential components of the β-catenin degradation pathway. Alterations of conductin function may lead to tumor formation.**

β-CATENIN: A PROTEIN WITH MULTIPLE FUNCTIONS

In mammals, β-catenin was originally identified as a component associated with cadherins, which are Ca^{2+}-dependent cell–cell adhesion molecules. β-Catenin and its relative plakoglobin (also termed γ-catenin) directly interact with the cytoplasmic tail of cadherins, but form mutually exclusive complexes. Via their amino-terminal domains, β-catenin and plakoglobin associate with the vinculin-related protein α-catenin, which in turn makes contact to actin filaments.[1] Catenins thus serve as a link between cadherins and the actin cytoskeleton (FIG. 1). Analysis of cytoplasmic de-

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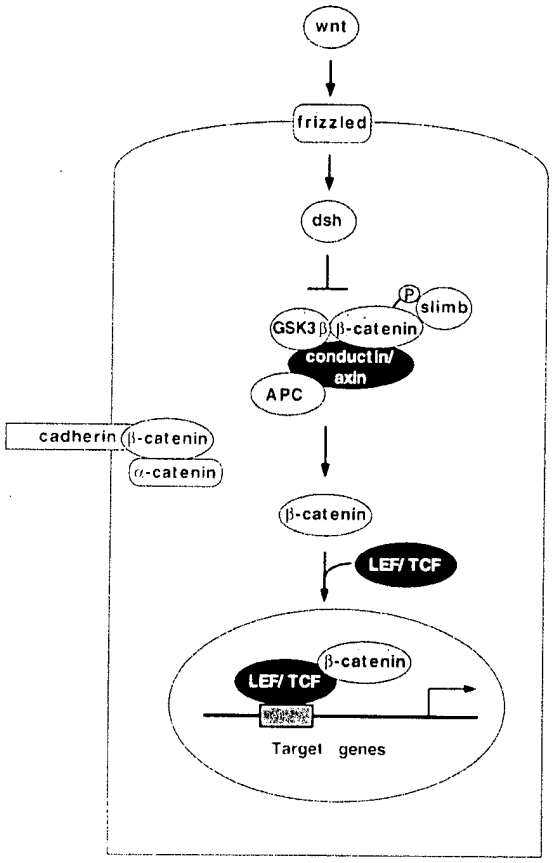
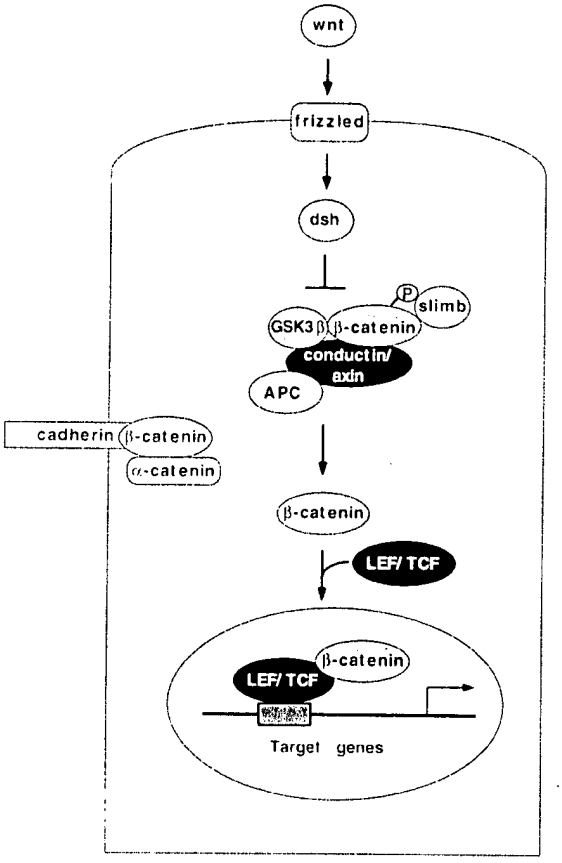


FIGURE 1. Schematic representation of the wnt signal transduction pathway. In the absence of wnts, β-catenin is phosphorylated by the conductin/axin complex and interacts with slimb (βTrCP), which mediates its ubiquitination and subsequent degradation. The block of the conductin/axin-based β-catenin degradation machinery by the wnt-frizzled-disheveled (dsh) cascade leads to the accumulation of cytosolic β-catenin, which then interacts with LEF/TCF transcription factors. The association of β-catenin with cadherin cell adhesion molecules and with α-catenin is also depicted. APC can also interact directly with β-catenin, which is not shown here. For further details, refer to the text.

letion mutants of cadherins and of cancer cell lines lacking specific catenins have revealed a crucial role of this association in cadherin-mediated cell adhesion.[2]

β-Catenin/armadillo also binds to numerous other partners in cadherin-independent complexes. The interactions with the tumor suppressor gene product APC, the LEF-1/TCF transcription factors, and the RGS domain proteins axin and conductin is important in the wnt signaling pathway (see below). Associations of β-catenin also have



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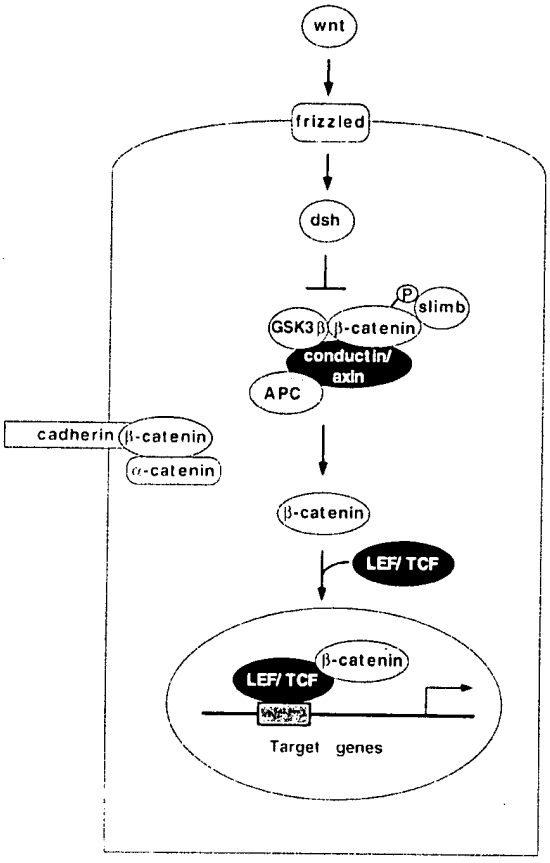

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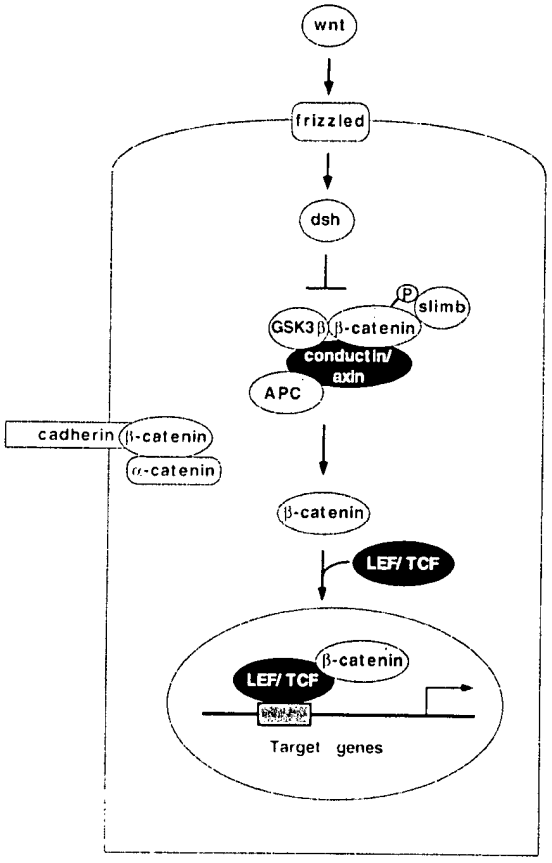

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been reported with the Alzheimer's disease protein presenilin,[3] and the TATA box binding protein pontin52.[4]

β-Catenin belongs to the armadillo family of proteins, which are characterized by a central domain of 12 repeats of about 40 amino acids (so-called arm-repeats).[5] This domain originally was described in armadillo, which is the *Drosophila* homologue of β-catenin. The structure of the central domain of β-catenin has been revealed by X-ray cristallography, which showed that the arm-repeats form a superhelix of helices.[6] A positively charged groove contained within the superhelix appears to represent the binding site for most of the interaction partners of β-catenin.

Besides its function in the cadherin complex, β-catenin has an essential role in the wnt signaling pathway (reviewed in Refs. 7–9). Genetic analyses in *Drosophila* showed that armadillo is a downstream component of wingless, the *Drosophila* homologue of wnt. Subsequently, β-catenin and homologues of it were shown to participate in wnt signaling in other organisms, such as mouse, chicken, *Xenopus*, zebrafish, and *C. elegans*. In *Xenopus* embryos, signaling through β-catenin is crucial for the determination of the body axis. Overexpression of β-catenin in ventral cells of the embryo leads to dorsalization of the derived structures and ultimately results in the formation of two-headed embryos. The same effects can be seen when activating members of the wnt pathway are expressed, and the *Xenopus* system (besides *Drosophila* genetics) has been instrumental in identifying the hierarchy of components of the signaling cascade.[10]

Wnt proteins are secreted factors that bind to transmembrane receptors of the frizzled family. This results in activation of the cytoplasmic phosphoprotein disheveled by an as yet unknown mechanism. Disheveled inhibits the activity of the conductin/axin-based β-catenin degradation complex, which in its active state catalyzes phosphorylation of β-catenin at specific residues in its N-terminal domain. This triggers ubiquitination of β-catenin and subsequent degradation by proteasomes. Inhibition of the β-catenin degradation complex by wnts leads to stabilization of cytosolic β-catenin, which then enters the cell nucleus and binds to transcription factors of the LEF/TCF family. As a result, transcription of specific target genes is activated by the TCF/β-catenin complexes (FIG. 1). Thus, stabilization of β-catenin appears to be the central molecular switch by which the wnt signal is transmitted to the cell nucleus.

THE β-CATENIN DEGRADATION MACHINERY

The control of cytoplasmic levels of β-catenin is an essential regulatory mechanism that prevents inappropriate transmission of signals to the nucleus in the absence of wnt factors. In nonstimulated cells, cytosolic β-catenin has a short half-live and is rapidly eliminated by proteasomes.[11] This is achieved by a highly complex β-catenin degradation machinery that counteracts accumulation of cytosolic β-catenin and apparently operates in a constitutive manner. When cells are stimulated by wnts, the degradation machinery is blocked and cytosolic β-catenin accumulates. It should be noted that degradation of β-catenin is restricted to the cytosolic pool of the protein and does not involve β-catenin bound to cadherins.

β-Catenin degradation appears to be initiated by the action of a multiprotein complex that is assembled by the core components conductin (also called axil) or axin.[12–15] The complex furthermore includes the serine/threonine kinase glycogen

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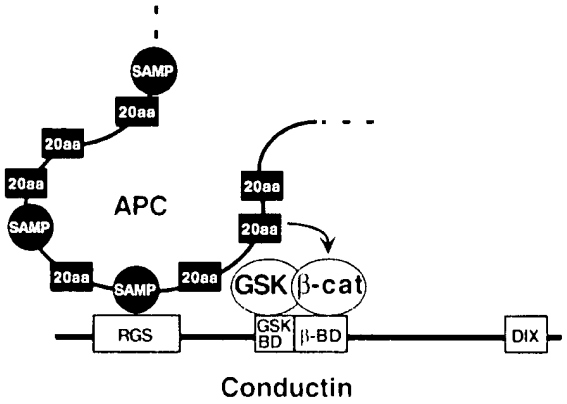

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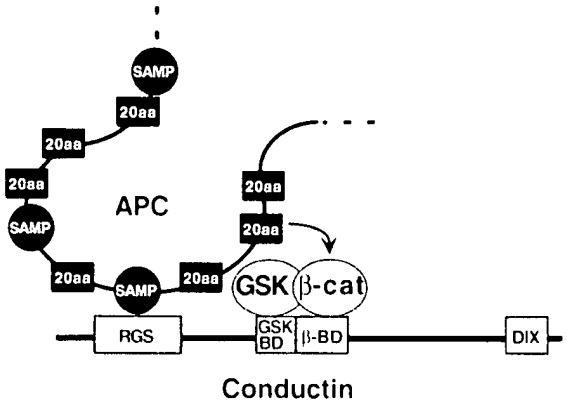

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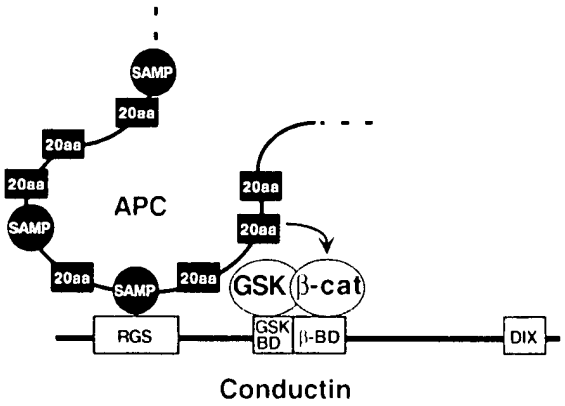

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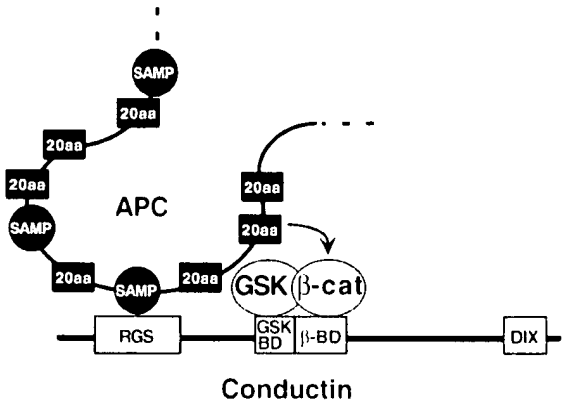

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synthase kinase 3β (GSK3β), the tumor suppressor gene product adenomatous polyposis coli (APC), and β-catenin, which all bind to conductin or axin at distinct sites (FIG. 2). In a yeast two-hybrid screen, we identified conductin as a specific interaction partner of β-catenin.[12] The murine conductin cDNA encodes a protein of 840 amino acids; it contains distinct binding sites for β-catenin and GSK3β in its center, a regulator of G-protein signaling (RGS) domain that binds to APC at the N-terminus, and a sequence related to disheveled at the C-terminus (FIG. 2). In β-catenin, arm-repeats 3–7 are responsible for conductin binding. The interaction of conductin with APC was detected in a yeast two-hybrid screen using the RGS domain as a bait. APC is a protein of 2845 amino acids built up by several domains, such as a heptad-repeat region, arm-repeat domains, 15 and 20 amino acid repeats, and a basic region. We found interacting protein fragments of APC, which encode three nonoverlapping parts of the 20 amino acid–repeat region. Analysis of these APC sequences revealed that all of them contain a repeat with the invariant core sequence ser-ala-met-pro (SAMP). The interaction between APC and conductin occurs via these SAMP elements, because mutation of SAMP to AALP abolished binding to conductin. Deletion analysis of conductin showed that the RGS domain is essential for binding to APC.[12]

Functional interaction of β-catenin and conductin was analyzed in SW480 colon carcinoma and Neuro2A cells. SW480 cells contain high levels of β-catenin due to mutation of the APC gene 3′ of codon 1338, which removes most of the 20 amino acids and all of the SAMP-repeats. As a consequence, β-catenin is located in the nucleus,

and this correlates with the activation of TCF/β-catenin–dependent promoters.[16,17] Introduction of wild-type APC leads to β-catenin degradation and suppresses TCF/β-catenin–dependent transcription (see also below). When we transiently expressed conductin in SW480 cells, β-catenin staining was lost in the transfected cells. Expression of conductin deletion mutants demonstrated that the β-catenin binding, but not the RGS domain, was required for β-catenin reduction. β-Catenin lacking the N-terminal phosphorylation sites required for induction of degradation was not affected by conductin. To interfere with conductin- or APC-mediated degradation, we employed dominant-negative mutants in Neuro2A cells, in which stability of endogenous β-catenin is low. Expression of a conductin mutant lacking RGS and β-catenin binding domains induced strong stabilization of endogenous β-catenin, indicating that this mutant interferes with endogenous conductin-mediated degradation of β-catenin. Expression of one of the conductin-binding fragments of APC also stabilized β-catenin, and point mutations of the SAMP-repeat in this fragment abolished stabilization of β-catenin.[12] We do not know precisely how the dominant-negative mutants work, but the conductin deletion mutant might form inactive heterodimers with endogenous conductin, and the fragments of APC might interfere with the endogenous conductin/APC interaction. Taken together the results indicate that conductin efficiently controls cytoplasmic levels of β-catenin, and that the interaction of conductin with APC is essential for degradation.

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nucleus. In epithelial cells endogenously expressed β-catenin was also translocated into the nucleus by transfected LEF-1.[38]

In the nucleus, LEF-1/TCFs bind to their target sequence via the HMG box, but apparently have no transcriptional activation function on their own. Rather, transcriptional regulation by the LEF/β-catenin complex is achieved through transcriptional activation domains that are present in the amino- and carboxy-terminal parts of β-catenin.[39–41] Moreover, we observed that β-catenin decreases the extend of LEF-1–induced DNA bending, indicating that the function of LEF-1 as an architectural transcription factor is modified.[38] Several target genes of the LEF-1/β-catenin complexes have been identified that all contain LEF/TCF binding sites. These include *ultrabithorax* in *Drosophila*, *nodal related 3* and *siamois* in *Xenopus*, and *c-myc* and cyclinD1 in humans.[42–48] It is of interest that β-catenin can also enter the nucleus independently of LEF-1 or TCFs in *in vitro* nuclear import assays.[49,50] β-catenin might therefore regulate nuclear functions independent of LEF-1/TCF.

To examine whether the LEF-1/β-catenin interaction plays a role in the wnt pathway, we analyzed the effect of the factors on axis formation in *Xenopus*.[38] Microinjection of LEF-1 mRNA into ventral blastomeres of *Xenopus* embryos induced the formation of a secondary axis, which was dependent on both the β-catenin-interaction and HMG domains. Coinjection of suboptimal amounts of β-catenin and full-length LEF-1 mRNAs revealed a synergistic effect of both factors on axis duplication. Importantly, injection of the LEF-1 mutant lacking the HMG domain into dorsal blastomeres resulted in ventralization of the *Xenopus* embryos.[38] This suggests that this mutant form of LEF-1 can act in a dominant-negative manner by interfering with the action of endogenous TCFs. Together, the data indicate that LEF-1 cooperates with β-catenin in embryonal axis induction in *Xenopus*. A role for TCF factors was also established in wingless signaling in *Drosophila*.

Certain members of the LEF/TCF family interact with groucho-related transcriptional repressors, which blocks activation of target promoters, as well as wnt signal transduction in *Xenopus* and *Drosophila*.[51,52] It is suggested that under physiological conditions the TCF/groucho complex actively represses gene transcription, which is overcome by activation of β-catenin. Interaction of *Drosophila* TCF was also shown with CBP (CREB-binding protein), which acetylates dTCF at its armadillo binding domain and antagonizes wingless signaling.[53]

THE wnt SIGNALING PATHWAY IN CANCER DEVELOPMENT

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In the nucleus, LEF-1/TCFs bind to their target sequence via the HMG box, but apparently have no transcriptional activation function on their own. Rather, transcriptional regulation by the LEF/β-catenin complex is achieved through transcriptional activation domains that are present in the amino- and carboxy-terminal parts of β-catenin.[39–41] Moreover, we observed that β-catenin decreases the extend of LEF-1–induced DNA bending, indicating that the function of LEF-1 as an architectural transcription factor is modified.[38] Several target genes of the LEF-1/β-catenin complexes have been identified that all contain LEF/TCF binding sites. These include *ultrabithorax* in *Drosophila*, *nodal related 3* and *siamois* in *Xenopus*, and *c-myc* and cyclinD1 in humans.[42–48] It is of interest that β-catenin can also enter the nucleus independently of LEF-1 or TCFs in *in vitro* nuclear import assays.[49,50] β-catenin might therefore regulate nuclear functions independent of LEF-1/TCF.

To examine whether the LEF-1/β-catenin interaction plays a role in the wnt pathway, we analyzed the effect of the factors on axis formation in *Xenopus*.[38] Microinjection of LEF-1 mRNA into ventral blastomeres of *Xenopus* embryos induced the formation of a secondary axis, which was dependent on both the β-catenin-interaction and HMG domains. Coinjection of suboptimal amounts of β-catenin and full-length LEF-1 mRNAs revealed a synergistic effect of both factors on axis duplication. Importantly, injection of the LEF-1 mutant lacking the HMG domain into dorsal blastomeres resulted in ventralization of the *Xenopus* embryos.[38] This suggests that this mutant form of LEF-1 can act in a dominant-negative manner by interfering with the action of endogenous TCFs. Together, the data indicate that LEF-1 cooperates with β-catenin in embryonal axis induction in *Xenopus*. A role for TCF factors was also established in wingless signaling in *Drosophila*.

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The APC protein can be subdivided in several domains, such as N-terminal heptad-repeats that mediate homooligomerization, armadillo-repeats of unknown function, and C-terminal domains that bind microtubules as well as other proteins, such as Dlg1 and EB1.[28] The central part of APC containing the 20–amino acid and SAMP repeats appears to be essential for downregulation of β-catenin.[17] The vast majority of mutations in APC in tumors occur in this region and result in C-terminally truncated APC proteins that in most cases still retain 20–amino acid repeats and bind to β-catenin, but lack all SAMP-repeats. Knockout mice of APC that retain the N-terminal part, including one SAMP-repeat, but miss all of the C-terminal domains, do not show any signs of tumor formation.[63] Together, this suggests that the interaction with axin or conductin is required for APC to function as a tumor suppressor and that both proteins must cooperate to mediate the degradation of β-catenin. However, it should be noted that both axin and conductin are capable of degrading β-catenin when overexpressed in cells that lack functional APC.[12,14] It is possible that under physiological conditions the interaction of APC and axin/conductin is required for the degradation of β-catenin, whereas under conditions of overexpression both proteins are sufficient for degradation. It is not yet understood which essential function APC contributes to the destruction complex.

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The generation of transcriptionally active TCF/β-catenin complexes in tumor cells results in the inappropriate activation of TCF target genes, including *c-myc* and cyclin D1,[42,44] which may ultimately lead to cancer. The expression of *c-myc* was found to be changed after induction of wild-type APC in an APC-deficient colon carcinoma cell line. Subsequent analysis showed that the *c-myc* promoter was activated by TCF-4/β-catenin via specific binding sites, and that expression of endogenous *c-myc* expression could be blocked by a dominant-negative TCF-4.[42] Because *c-myc* overexpression previously had been linked to colorectal tumorigenesis, it is possible that TCF-4/β-catenin induces neoplastic growth via activation of *c-myc*. It is further indicated in this study that several other genes that might contribute to tumorigenesis are up- or downregulated by APC. Components of the *c-jun* family and the metalloproteinase matrilysin were also shown to be targets of TCF/β-catenin.[45,46]

TCF/β-catenin complexes were shown to have transforming activity in some cell types.[65,66] Analysis of transgenic and knockout animals has revealed further aspects of β-catenin signaling in tumor formation. Mice deficient in the β-catenin partner TCF-4 showed lack of proliferation of the stem-cell compartment in the prospective crypts of the small intestine, indicating that activation of TCF/β-catenin as observed in colorectal tumors leads to uncontrolled growth of stem cells.[67] In line with this, mutations in APC appear early during colon carcinogenesis and are associated with the development of adenomas.[59] Interestingly, knockout mice deficient for TCF-1 were recently shown to develop adenomas in the gut and the mammary glands.[68] This indicates that TCF-1 acts as a repressor of oncogenic target genes in the absence of activated β-catenin (possibly through interaction with grouchos), and that depletion of TCF-1 in the knockout animal results in the relief of this repression and cellular transformation.

Deletion of parts of the amino terminus of β-catenin has been shown to increase its stability and convert it to a weak oncogene *in vitro*. Expression of similar deletion mutants in transgenic animals led to increased cell proliferation in intestinal crypt cells, which was accompanied by an increase in apoptosis of these cells.[69] In a recent study, the GSK3β target sites in the N-terminus of β-catenin were mutated in the intestinal epithelium of mice by cre/loxP-mediated homologous recombination. The mice developed multiple adenomatous intestinal polyps, demonstrating that activation of β-catenin is involved in intestinal tumorigenesis.[70] Tumor formation was also induced by expression of stabilized β-catenin in the epidermis of mice. Benign hair follicle tumors developed that resemble specific human skin tumors that contain β-catenin mutations.[71,72] Collectively, the results indicate that stabilized versions of β-catenin have oncogenic capacity *in vivo*.

It is of note that the cadherin-based cell–cell adhesion is frequently disturbed in tumors (reviewed in Ref. 64). The epithelial E-cadherin is often downregulated in transformed epithelial cells and human carcinoma cells, which correlates with a dedifferentiated phenotype and increased invasiveness *in vitro*. Forced expression of E-cadherin blocks invasiveness of tumor cells *in vitro* and even prevents tumor progression and metastasis formation in *in vivo* model systems. Moreover, mutations of the E-cadherin gene have been identified in lobular breast and gastric carcinomas. One might speculate that loss of cadherins leads to the release of oncogenic β-catenin

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Deletion of parts of the amino terminus of β-catenin has been shown to increase its stability and convert it to a weak oncogene *in vitro*. Expression of similar deletion mutants in transgenic animals led to increased cell proliferation in intestinal crypt cells, which was accompanied by an increase in apoptosis of these cells.[69] In a recent study, the GSK3β target sites in the N-terminus of β-catenin were mutated in the intestinal epithelium of mice by cre/loxP-mediated homologous recombination. The mice developed multiple adenomatous intestinal polyps, demonstrating that activation of β-catenin is involved in intestinal tumorigenesis.[70] Tumor formation was also induced by expression of stabilized β-catenin in the epidermis of mice. Benign hair follicle tumors developed that resemble specific human skin tumors that contain β-catenin mutations.[71,72] Collectively, the results indicate that stabilized versions of β-catenin have oncogenic capacity *in vivo*.

It is of note that the cadherin-based cell–cell adhesion is frequently disturbed in tumors (reviewed in Ref. 64). The epithelial E-cadherin is often downregulated in transformed epithelial cells and human carcinoma cells, which correlates with a dedifferentiated phenotype and increased invasiveness *in vitro*. Forced expression of E-cadherin blocks invasiveness of tumor cells *in vitro* and even prevents tumor progression and metastasis formation in *in vivo* model systems. Moreover, mutations of the E-cadherin gene have been identified in lobular breast and gastric carcinomas. One might speculate that loss of cadherins leads to the release of oncogenic β-catenin

that modulate the transcriptional activity of the LEF-1/TCF-β-catenin complexes might be altered in tumors.

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DISCUSSION

S.H. ITZKOWITZ (*Mount Sinai School of Medicine, New York*): The recent data indicate that TCF1 may be downstream from TCF4. How does this tie in with the β-

catenin story? Does β-catenin also bind TCF1 and, if so, is it the same way as it does with TCF4?

BEHRENS: The point you have mentioned is quite complicated. The TCFs can also act as transcriptional repressors, at least some of them. For instance, TCF1 binds to transcriptional repressors such as groucho,[52] so it can actively repress transcription. It was shown that the knockout of TCF1 actually leads to increased tumor formation in mice. This is because the repressor function of TCF1 is lost, and TCF4 comes in and interacts with β-catenin. There is one interesting twist to the system, because on the one hand, TCFs are needed for transcriptional activation in the presence of β-catenin, but in the absence of the latter, they can act as repressors. The main switch appears to be this change from a repressor to an activator function, induced by β-catenin. Therefore TCF1 might turn out to be a kind of tumor suppressor and might be mutated in certain tumors.

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S.I. REED (*The Scripps Research Institute, La Jolla, CA*): Do you have any idea what the target genes of LEF/TCF are? Are they involved in tumorigenesis?

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BEHRENS: We have specific mutants of β-catenin that do not bind to conductin, but can still interact with APC. Whether the affinity of these mutants is increased or decreased, we do not know. We think that the components of this complex may regulate each other's affinity, but we do not know the order of events, that is, which component binds first and which comes then, but it could well be that, for instance, binding of APC to conductin could increase the affinity of conductin to β-catenin. On the other hand, it was shown that phosphorylation of conductin or axin by GSK3β affects the affinity to β-catenin.[26] So the complex formation is probably highly regulated, and the different components might influence each other.

B. MANN (*Freie Universität Berlin, Berlin*): Did you find any evidence that conductin is involved in colorectal carcinogenesis? Did you check cell lines or patients' tissues for conductin difference in expression or mutations?

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Expression of the *int*-1 Gene in Transgenic Mice Is Associated with Mammary Gland Hyperplasia and Adenocarcinomas in Male and Female Mice

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Summary

Transcriptional activation of the *int*-1 gene by proviral insertion mutations is thought to be a key step in mammary tumor induction by the mouse mammary tumor virus (MMTV). To test this hypothesis, we have constructed an *int*-1 allele resembling those found in virus-induced tumors, with an MMTV LTR placed 5′ to the *int*-1 gene in the opposite transcriptional orientation. Transgenic mice harboring this allele express *int*-1 RNA at high levels in mammary and salivary glands of male and female mice and in male reproductive organs. The mammary glands of males and virgin females are grossly hyperplastic compared with those of nontransgenic littermates. Mammary and (less frequently) salivary adenocarcinomas occur in these animals at rates indicating that transcriptional activation of *int*-1 and associated hyperplasia are initiating events in multistep carcinogenesis.

Introduction

Many retroviruses lacking viral oncogenes appear to initiate neoplasia by causing proviral insertion mutations that augment transcription of adjacent proto-oncogenes (Varmus, 1982). Several proto-oncogenes have now been identified as targets for insertion mutations by these viruses (Varmus, 1987), but for many of these genes, proposed roles in oncogenesis are based largely upon the circumstantial evidence of frequent mutations in one type of tumor.

The *int*-1 gene was the first to be implicated in tumorigenesis on this basis, when it was discovered to be the target for mouse mammary tumor virus (MMTV) proviral insertions in the majority of virus-induced mammary carcinomas in C3H mice (Nuss and Varmus, 1982). At least three other unrelated genes (*int*-2, *int*-3, and *int*-4) also appear to serve as targets for insertion mutations during MMTV-mediated carcinogenesis (Nusse, 1988). The *int*-1 gene appears not to be expressed in normal mammary glands from pregnant or lactating mice (Nusse and Varmus, 1982; Nusse et al., 1984) nor in any other tissues save the neural tube in midgestational embryos and the tests (early spermatids) in mature males (Jakobovits et al., 1986; Shackleford and Varmus, 1987; Wilkinson et al., 1987). Proviral insertions are associated with the appearance of one to ten copies per cell of *int*-1 RNA in mammary tumors (Nusse et al., 1984). These insertions reside outside the *int*-1 coding domains, usually either upstream of the gene in the opposite transcriptional orientation or downstream in the same orientation, implying that the MMTV provirus supplies an enhancer element, rather than a promoter, to cause ectopic production of normal *int*-1 protein (Nusse et al., 1984; van Ooyen and Nusse, 1984).

The claims that such insertion mutations are instrumental in mammary tumorigenesis and that *int*-1 is therefore a proto-oncogene are supported by studies of the gene in cultured cells. Although it is unable to affect the behavior of fibroblasts, *int*-1 induces striking alterations of morphology and growth potential in two established mammary cell lines when expressed from the promoter in a murine leukemia virus (MLV) long terminal repeat (LTR) (Brown et al., 1986; Rijsewijk et al., 1987).

To obtain a more accurate assessment of the pathogenic role of *int*-1, however, it is necessary to express the gene in normal mammary epithelial cells, preferably in the intact animal where other postulated oncogenic factors—genetic, hormonal, and viral—can also be evaluated. We have therefore generated transgenic mice that carry the putative initiating lesion, an *int*-1 gene activated by the MMTV enhancer, in all mammary cells, not just in the rare cell with an appropriate proviral insertion, as occurs in virus-infected animals.

In the transgenic line we have studied most extensively, a fabricated *int*-1 allele resembling alleles found in virus-induced tumors is expressed in mammary and salivary glands and male reproductive organs. The mammary glands respond by marked proliferation of alveolar and ductal epithelium in both males and females, without manipulation of hormonal status. Adenocarcinomas occur frequently in female and occasionally in male mammary glands, as well as in salivary glands. The appearance of hyperplastic glands prior to tumor formation in these mice suggests that *int*-1 gene products initiate proliferation of at least mammary and possibly salivary epithelial cells and that this represents the first stage in multi-step carcinogenesis.

Results and Discussion

Generation of Transgenic Mice

To produce mice with a potentially tumorigenic *int*-1 transgene, we utilized a molecular clone in which the MMTV LTR was positioned about 1 kilobase (kb) upstream of a genomic clone of *int*-1. The arrangement of the LTR within this clone approximates that observed in several *int*-1 alleles in virus-induced tumors (Figure 1A). However, the transgene differs in two ways from tumor-associated alleles. First, most of the MMTV provirus is absent. As a result, none of the known viral genes, including the long

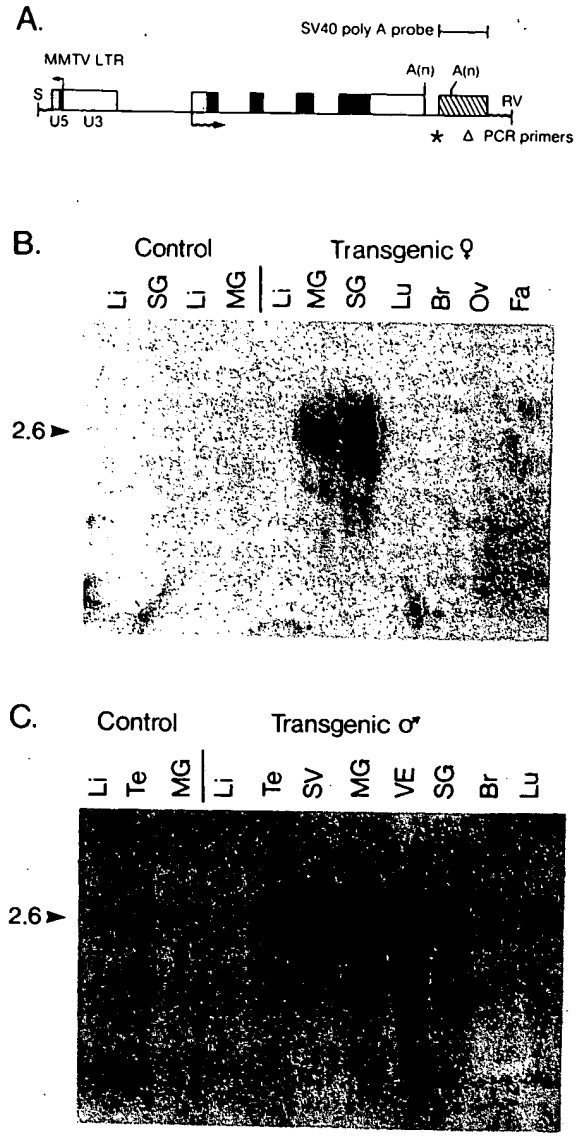

Figure 1. Structure and Expression of the *int*-1 Transgene

(A) The transgene. The MMTV enhancer-driven *int*-1 gene was constructed by placing the MMTV-LTR approximately 1.0 kb upstream of the genomic *int*-1 gene, such that transcription from the LTR is in the opposite transcriptional orientation. The exons are shown as boxes, with the coding regions filled in. The MMTV-LTR is an authentic viral LTR obtained from the C3H MMTV virus. It contains all but approximately 30 bp from each end. An 850 bp fragment containing the SV40 splice and polyadenylation sites (cross-hatched box) was added downstream of the *int*-1 polyadenylation site. The plasmid was digested with the restriction endonucleases SalI (S) and EcoRV (RV) to generate the fragment shown. The wavy arrow represents the *int*-1 transcription initiation site (van Ooyen and Nusse, 1984; J. Mason, personal communication). The bar above the SV40 sequence represents the fragment used as the hybridization probe for Southern and Northern blots. The star (*) and triangle (Δ) below the line represent the sites of the oligonucleotides used for priming the polymerase chain reaction. (B) Northern blot analysis of female RNA and (C) Northern blot of male RNA. Total cellular RNA (10 μg) was isolated from various tissues and separated onto agarose formaldehyde gels, blotted to a nylon membrane, and hybridized with ^{32}P-labeled probes for *int*-1 as described in Experimental Procedures. Liver (Li), salivary gland (SG), mammary gland (MG), lung (Lu), brain (Br), ovary (Ov), fat (F), testes (Te), seminal vesicles (SV), and vas deferens/epididymis (VE).

open reading frame in the LTR (Donehower et al., 1980), can be express d from the transgene. Thus, the viral elem nt is likely to contribute only its glucocorticoid-responsive enhancer to the cloned gene. Second, a small fragment of Simian virus 40 (SV40) DNA has been inserted downstream of the final exon of *int*-1 to provide an additional polyadenylation site and a marker to distinguish the transgene from the endogenous *int*-1 gene. An authentic, activated *int*-1 allele cloned from a virus-induced tumor was not used because of the difficulty of cloning a region of MMTV proviral DNA that lies near the 5′ LTR (Donehower et al., 1980; Majors and Varmus, 1981) and hence adjacent to the *int*-1-proximal LTR in the vast majority of activated alleles (Nusse and Varmus, 1982; Nusse et al., 1984).

Before introducing the *int*-1 transgene into male pronuclei of fertilized eggs from (C57BL/6 × SJL) F1 mice, it was freed of most plasmid sequences by digestion with EcoRV and SalI, producing a 7 kb linear DNA (Figure 1A). Tail DNA from seven weanling mice derived from injected eggs was analyzed by hybridization with a labeled SV40 DNA probe. One male (mouse #7) contained multiple tandem copies of the transgene, apparently located at a single integration site (data not shown). Mouse #7 served as the founder animal for the single line of *int*-1 transgenic mice (line 303) that we have studied most comprehensively. The transgene was transmitted in a simple Mendelian pattern (data not shown), as monitored directly by hybridization with a transgene-specific probe or following amplification of the transgene in tail or white blood cell DNA by the polymerase chain reaction (see Experimental Procedures and the legend to Figure 1A). The genealogy provided further support for the conclusion that the transgenic DNA is located at a single chromosomal site in line 303 mice, though we cannot exclude multiple insertions on a single chromosome.

Expression of the *int*-1 Transgene

Total RNA was prepared from several organs from male and female transgenic mice to measure the steady-state concentration of *int*-1 RNA. The RNA was gel fractionated by size and hybridized with a labeled *int*-1 probe after transfer to nylon membrane. Relatively high levels of the expected 2.6 kb *int*-1 RNA were detected in samples from mammary and salivary glands from female mice (Figure 1B). High levels of expression in these organs are consistent with earlier findings in MMTV-infected mice (Nandi and McGrath, 1973; Hilgers and Bentvelzen, 1978; Teich et al., 1984) and in transgenic mice in which the MMTV LTR was used as a promoter for other genes (Stewart et al., 1984; Leder et al., 1986; Sinn et al., 1987; Stewart et al., 1988).

Samples from male transgenic mice also revealed r latively high l vels of *int*-1 RNA in salivary glands (Figure 1C). Again consistent with earli r studi s of transgenic mic and MMTV-inf cted animals, *int*-1 RNA was abundant in the mal reproductive tract (testis, seminiferous tubules, and vas deferens). As previously reported, the ndogenous *int*-1 gene is express d in th normal testis

(Jakobovits et al., 1986; Shackleford and Varmus, 1987); however, s veral-fold higher levels of *int*-1 RNA were observed in the transg nic testis, implying expression of the transgene in unidentified testicular cells. Low levels of *int*-1 RNA were observed in samples from spleen, heart, brain, and lymphoid tissues in both females and males (Figures 1B and 1C and data not shown).

Because of our particular interest in the effect of *int*-1 on mammary cells, we attempted to measure *int*-1 RNA in the fat pads of male mice, which normally harbor only a few rudimentary ducts from the mammary gland anlage. Surprisingly, the levels of *int*-1 RNA in fat pads from the transgenic males (Figure 1C) were as high as those observed in the mammary glands of virgin or multiparous female transgenics (Figure 1B, also Figure 5 below). This observation prompted further anatomical studies of the mammary glands of the male transgenics, as described in the next section.

Normal mice were previously shown to lack detectable amounts of *int*-1 RNA in any of the organs studied here, save testes (Jakobovits et al., 1986; Shackleford and Varmus, 1987), and we have obtained similar results with RNA from nontransgenic siblings of transgenic animals (Figures 1B and 1C). Thus, we assume that the *int*-1 RNA shown in Figure 1 reflects expression of the transgene, rather than activation of the endogenous *int*-1 gene. Further support for this conclusion comes from treatment of the transgenic mice with hydrocortisone: a 2- to 3-fold increment in *int*-1 RNA occurred (data not shown), suggestive of expression under the control of the glucocorticoid-responsive MMTV LTR, rather than from the endogenous gene. Because the *int*-1 polyadenylation site seems to have been efficiently used during expression of the transgene, we were unable to use SV40 sequences to distinguish transgenic and endogenous transcripts.

Mammary Glands from *int*-1 Transgenic Males and Females Are Hyperplastic

We devoted particular attention to the status of the mammary gland in apparently normal transgenic animals for several reasons. First, prior to these studies, the mammary gland was the only known target for oncogenic effects of *int*-1. Second, experiments described in the preceding section showed that the MMTV-*int*-1 transgene is efficiently expressed in mammary glands. Third, MMTV-induced mammary carcinoma is frequently preceded by the appearance of hyperplastic alveolar nodules, a presumptive preneoplastic lesion (DeOme et al., 1959; Medina, 1982). Finally, transgenic females were unable to deliver milk to their young. The lactation defect was first suspected when all pups born to transgenic females died within th first day of life with little if any milk in their stomachs. Subsequent offspring surviv d without difficulty if th y were foster-nursed on normal femal s; unsuccessful attempts to obtain milk with a mechanical milking device confirmed that the transgenic females are unable to produc milk in a normal fashion.

To insp ct th mammary glands anatomically, whol mounts of fat pads from transgenic and control litt rmates wer prepared (Figure 2). Mammary glands from nontransgenic virgin females consist of extensive ducts, with a few alveoli growing from terminal branches at the end buds of the ducts (Figure 2A). In contrast, glands from transgenic virgin females resemble the hormonally stimulated glands normally observed in pregnant animals: there is a marked increase in the number of terminal branches and alveoli, producing a diffuse lobular hyperplasia (Figure 2B).

The findings with fat pads from male mice were even more striking. As previously described for most other strains of mice (Staff of the Jackson Laboratory, 1966), fat pads from our nontransgenic males contain only a few simple ducts and branches, without end buds or alveoli (Figure 2C). Whole mounts of 13 fat pads from seven transgenic males showed extensive lobular-alveolar development (Figure 2D), similar to the pattern observed in the transgenic virgin females. There are, however, two obvious differences between the fat pads of the transgenic males and females: the female glands are larger, filling the fat pad, whereas the male glands occupy 20%–80% of the fat pad; and there appears to be more lobular-alveolar structures in the transgenic male gland compared with the female.

Lobular alveolar hyperplasia of the sort observed in our male and female transgenic mice is similar to that observed in normal females during pregnancy and lactation and in virgin females subjected to appropriate hormonal treatment. In males, estrogen treatment, with or without orchiectomy, results in feminization and renders these animals more susceptible to mammary tumor formation (Moore, 1975). In our virgin females and males, however, no hormonal therapy was required to induce mammary hyperplasia. The male mice showed no other evidence of feminization; indeed, affected males were fertile and fully active sexually, as best demonstrated by their ability to sire multiple litters. It would appear that expression of the *int*-1 transgene can supercede the hormonal signals that normally govern mammary gland involution in males and delay ductal and alveolar proliferation until pregnancy in females.

Thus, expression of the *int*-1 transgene in mammary cells is associated with marked alveolar and ductal hyperplasia in both male and female mammary glands, without a requirement for pregnancy in the females or feminizing hormones in the males. Our animals may provide a useful model for further assessing endocrine control of mammary development.

Mammary Carcinomas Occur in Both Female and Male Transgenic Mice

In accord with the prediction that expression of *int*-1 causes or strongly predisposes to mammary carcinogenesis, many of the mice in the transgenic line 303 hav developed adenocarcinomas of the br ast. Among the females, 3 of 19 virgin mice and all of 5 breeding mic surviving to the ag of 4 months developed such tumors. By the age of 7 months, ov r 80% of the females had d - veloped tumors, regardless of breeding status (Figure 3).

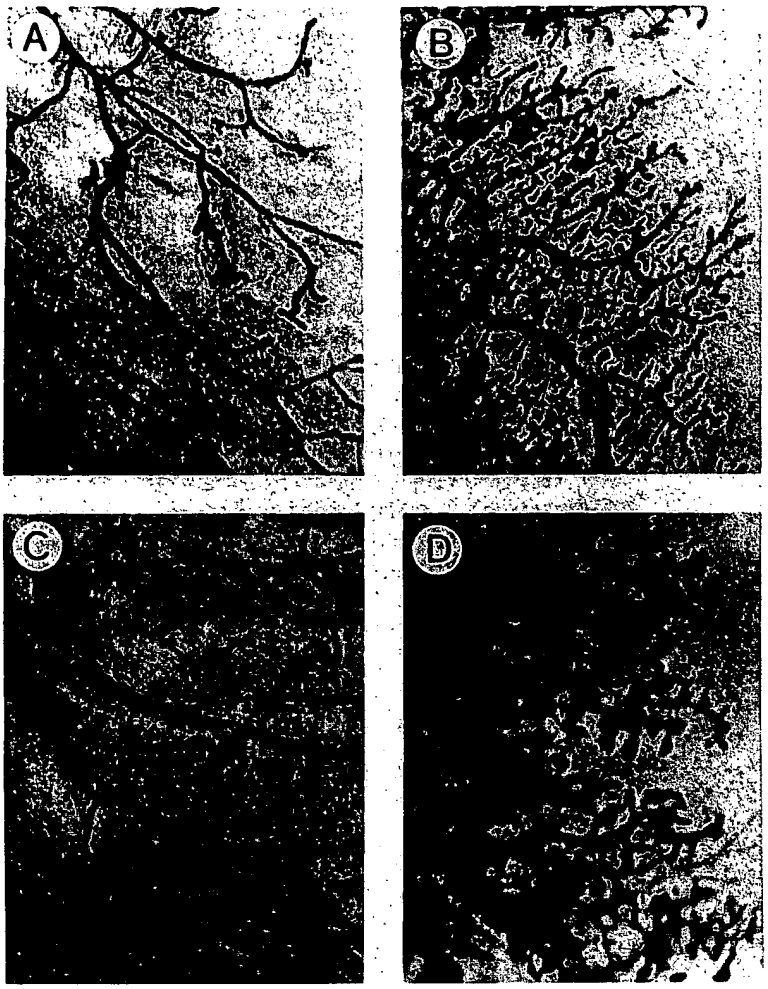

Figure 2. Wholemount Analysis of Control and Transgenic Mammary Glands

(A) and (C) are whole mounts of mammary glands from a control female and male mouse, respectively. (B) and (D) are mammary glands from a transgenic female and male. Magnification is 19×. Note the extent of lobular-alveolar growth in transgenic animals compared with controls.

When initially examined, the animals had a single tumor. To determine whether additional tumors would develop in other mammary glands, small tumors (less than 1 cm in diameter) were surgically removed from six animals. All of the postoperative mice developed one or more additional tumors in other glands within 1–2 months.

Mammary tumors have also been observed in two male transgenic mice, at the ages of 3 and 8 months. Thus far, nine males have been maintained to the age of 10 months without the appearance of tumors, suggesting that the tumor incidence is lower in males than in virgin females. Both of the tumor-bearing males appeared to be hormonally normal, and one sired multiple litters.

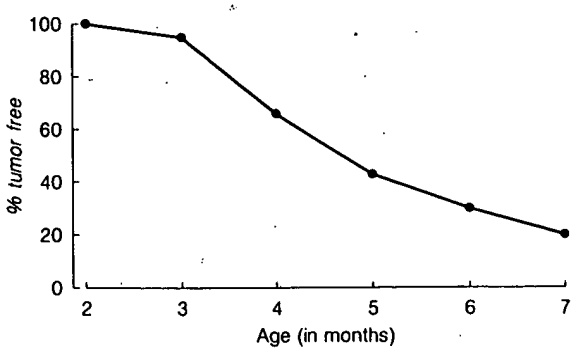

Figure 3. Tumor Incidence in Female of Line 303

The percentage of animals, both virgin and breeding, that remain tumor free is plotted as a function of age in months.

Histological examination of female and male mammary tumors was most consistent with a diagnosis of mammary adenocarcinoma, indistinguishable from MMTV-induced disease (Figure 4 and data not shown). The samples showed heterogeneous glandular structures, with frequent blood- or secretion-filled cysts at medium magnification (data not shown). At higher magnification, cytological abnormalities characteristic of malignancy are readily observed: high nuclear:cytoplasmic ratios, hyperchromatic nuclei, cellular pleomorphisms (Figure 4A), and numerous mitotic figures were present in some areas.

Although metastasis is uncommon in MMTV-induced tumors, we occasionally obs rved metastatic lesions in transgenic females. For exampl , metastatic involvem nt of a noncontiguous c rvical lymph nod occurred in one animal with a mammary tumor 3 cm in diam ter. Carcinomatous cells had invaded the subcapsular sinus of the nod (Figur 4B), a common sit of ntry of metastatic tumors.

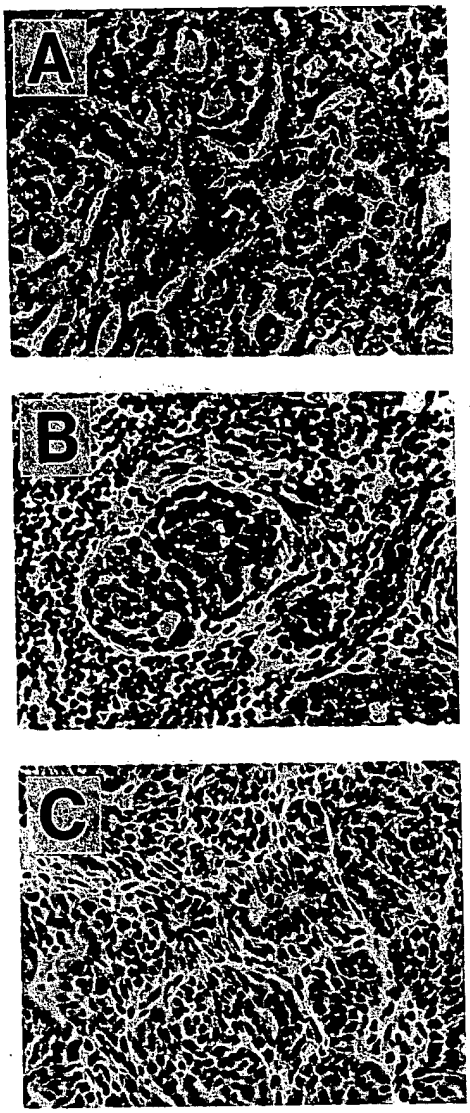

Figure 4. Histological Sections of Tumors Arising in Line 303 Mice

(A) High power view of a mammary adenocarcinoma from female 303–30. (B) High power view of an island of adenocarcinoma within a lymph node in female 303–37 with a gross mammary adenocarcinoma. (C) High power view of a salivary tumor in female 303–29. Original magnification is 220×.

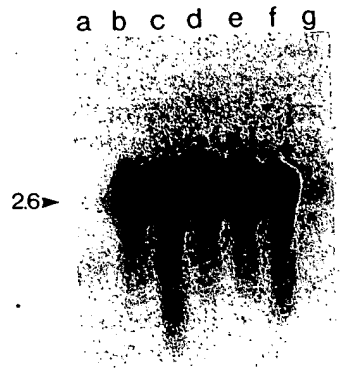

Figure 5. *int*-1 RNA in Mammary Tissues in Line 303 Females

Thirty micrograms of total cellular RNA was prepared and analyzed with an *int*-1 probe as described in Figure 2. Lane a, (C57B1/6 × SJL)F_1 testes; lane b, 303–29 normal mammary gland; lane c, 303–29 mammary tumor; lane d, 303–29P1 tumor: cells derived from tumor 303–29 were transplanted into a parental F_1 host that subsequently developed a tumor; lane e, 303–30 mammary tumor; lane f, 303–30 normal mammary gland; and lane g, 303–9 transgenic testes RNA.

To show that the mammary tumors continue to express the *int*-1 transgene, we examined RNA from primary and transplanted mammary tumors and from normal mammary glands from tumor-bearing mice for *int*-1 transcripts. No significant differences in concentration of the 2.6 kb *int*-1 mRNA were found among sampl s from two transgenic animals (Figure 5). We conclude that inheritanc and expression of the activated *int*-1 transg ne ar associated with the appearance of malignant adenocarcinoma in our animals and that the tumors arise from *int*-1 RNA-positive cells in the hyperplastic mammary glands.

We feel justified in drawing strong conclusions from the study of this singl line for three reasons. First, the phenotype in line 303 is inherited in a dominant fashion and exhibited by both females and males, hence not requiring homo- or hemizygosis. Second, we have recently produced seven new founder mice with an MMTV-*int*-1 transgene; at least one of the female founders is unable to deliver milk to her young, and four of five female founders display the mammary gland lobular-alveolar hyperplasia seen in line 303. Third, the strong similarities between the pathogenetic features of MMTV-induced and transgene-associated diseases argue strongly for a causative association between the transgene, its expression, and proliferative abnormalities of the mammary epithelium.

Salivary Adenocarcinomas Also Occur In MMTV-*int*-1 Transgenic Animals

Although *int*-1 has been implicated as an oncogene only in mammary tumors in virus-infected animals, it seemed possible that ectopic expression of *int*-1 in other organs, especially those composed of glandular epithelium, might also be tumorigenic. This prediction has been confirmed by the appearance of salivary tumors in one female and one male transgenic animal in line 303. Spontaneous salivary gland tumors are uncommon in laboratory mice and have not been observed in any of our nontransgenic control animals. In addition, the most common spontaneous salivary tumors in mice are myoepitheliomas (Dawe, 1979), whereas the tumors in the *int*-1 transgenic mice are adenocarcinomas, with many of the histological features also seen in the mammary tumors (Figure 4C). Thus, it is likely that the app arance of salivary as well as mammary tumors depends upon the presence and x- pr ssion of the *int*-1 transg ne.

The Oncog nic Pot ntial of the *int*-1 Gene

Although our transg nic experiments xtend th oncogenic potential of *int*-1 to salivary as well as mammary glands, it appears that *int*-1 is less potent as an oncog ne

in the salivary gland and that high levels of expression on the gene in male reproductiv organs has no obvious neoplastic or teratogenic consequences. This is consistent with earlier reports that introduction of an active *int*-1 gene into a number of cultured cell lines, e.g., fibroblasts, has no apparent effects on growth or morphology (Brown et al., 1986; Rijsewijk et al., 1987). Such observations might b explained by a deficiency of surface receptors for the int-1 protein (see below) or by an inability of certain cell types to respond to signaling by int-1 protein.

Our results strongly suggest that expression of the *int*-1 gene in mammary and salivary gland epithelium is a causative factor in the frequent appearance of malignant tumors in those organs. The abundance of *int*-1 RNA in precancerous glands (Figures 2 and 5) and the diffuse hyperplasia observed in mammary glands imply that most if not all cells in the glands of transgenic animals express the gene; nevertheless, only one or a few of the ten mammary glands are likely to develop tumors, even by six months of age. Thus, additional events, perhaps mutations involving as yet unknown proto-oncogenes, must be required to achieve a neoplastic phenotype. Thus, we argue that *int*-1 is a mammary and salivary oncogene but contributory rather than sufficient.

Similar arguments have been made about B cell lymphomas, mammary tumors, and other neoplasms arising in transgenic mice constructed with c-*myc*, the SV-40 early gene, and other oncogenes. In several of these oncogene-b aring transgenic lines, preneoplastic hyperplasias have been described and deemed to be the prelude to frank tumors (Cory and Adams, 1988). We also describe here a premalignant state for mammary glands. As in those reported cases, the mice described here provide a useful point of departure for attempting to isolate the genes involved in postulated secondary events in neoplasia. One advantage of our *int*-1 transgenic mice is that the transgene simulates tumor-associated alleles found naturally in MMTV-induced carcinomas; hence, any putative collaborating oncogene isolated in the transgenic model can also be examined in the context of virus-induced disease.

The contribution made by *int*-1 to mammary (and perhaps salivary) carcinogenesis appears to be accelerated proliferation of the alveolar epithelium in our transgenic mice. We also have provisional evidence for similar effects in primary mouse mammary epithelial cultures infected with MLV vectors carrying the *int*-1 gene. When such cells are introduced into cleared fat pads of syngeneic animals, they form hyperplastic alveolar nodules (HAN), similar to those observed in the preneoplastic glands of MMTV-inf cted females (unpublished data of A. Tsukamoto, U. Ehmann, R. Guzman, and H. E. Varmus).

The rate of appearance of mammary tumors in our transg nic mice does not differ markedly from that commonly observed in MMTV-infected mice (Nandi and McGrath, 1973; Moore, 1975; Hilg rs and Bentvelz n, 1978). This may seem surprising since all mammary cells in the transg nic animals probably expr ss *int*-1 and h nce are at high risk of oncog nic conversion, whereas xpression of *int*-1 in virus-infected mice is dependent upon ins rtion mutations that ar presumed to be rare events. This apparent paradox might be explained in sev ral ways. For example, the number of cells expressing *int*-1 in a preneoplastic mammary gland in a virus-infected animal might be higher than expected, due to the proliferative advantage conferred by activation of the gene or due to an unexpected preference for integration in the *int*-1 locus. Alternatively, MMTV itself might contribute a function, e.g., encoded in the long open reading frame in the LTR, that accelerates tumorigenesis. These possibilities are currently under investigation.

Conclusion

We have produced transgenic mice carrying an *int*-1 allele that resembles alleles observed in MMTV-induced mammary carcinomas. These animals exhibit high levels of *int*-1 RNA in mammary and salivary glands in males and females, and expression is associated with marked hyperplasia of the mammary glands and with the appearance of adenocarcinomas in both types of glandular epithelium. Thus, we have generated a mouse line that transmits as a dominant genetic trait a lesion usually acquired only sporadically in virus-infected somatic cells. These mice validate the classification of *int*-1 as a proto-oncogene, provide an appropriate setting for determining the oncogenic and developmental effects of ectopic expression of *int*-1, and offer experimental opportunities for seeking additional steps in the pathogenesis of MMTV-induced tumors.

Experimental Procedures

Transgene Construction and Generation of Transgenic Mice

The enhancer-driven *int*-1 gene was constructed from a plasmid, M5.3–12, obtained from G. Shackleford. This plasmid contains the entire *int*-1 transcriptional unit placed about 1 kb downstream from an MMTV-LTR. To this clone we have added the splice and polyadenylation sites contained on an 850 bp BamHI–BglII fragment of SV40 DNA (Mulligan and Berg, 1981). This fragment was inserted at the BglII site at the 3′ end of the *int*-1 gene. The EcoRV to SalI fragment (approximately 7 kb) was isolated for microinjection by preparative agarose gel electrophoresis and GENECLEAN elution. The DNA was further purified and injected as described (Hogan et al., 1986). Three weeks after birth, total genomic DNA was isolated from tail samples from the pups. Southern blotting was performed on BamHI-digested genomic DNA and probed with the 850 bp SV40 poly(A) fragment (Figure 1). Animals testing positive for the transgene were used for further breeding and analysis. Subsequent testing of progeny was done by the polymerase chain reaction using DNA isolated from whole blood (R. Higuchi, Cetus Corp., personal communication).

RNA Extraction and Northern Blots

Total cellular RNA was prepared by Polytron homogenization of cells as previously described (Shackleford and Varmus, 1987). Total RNA was electrophoresed, transferred to GeneScreen nylon membranes, and hybridized according to Shackleford and Varmus (1987).

The *int*-1 hybridization probe was a full-length cDNA fragment isolated from an SP65 plasmid containing the *int*-1 cDNA (Brown et al., 1986). Gel-purified fragments were ^{32}P-labeled by nick-translation using the standard procedures (Maniatis et al., 1982).

Whole Mount and Histological Analysis

Inguinal or thoracic mammary fat pads were removed from positive and control animals under general anesthetics (avertin; Hogan et al., 1986). Each fat pad was spread in a Tissue Tek capsule, fixed in Tellyesniczky's fixative, defatted in acetone, and stained in iron hematoxylin. These whole mounts were analyzed in methyl salicylate under a dissecting microscope.

Tumors were excised from anesthesized mice, fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histological examination.

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Secreted Molecules in Metanephric Induction

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Abstract. Nearly 50 yr ago, Clifford Grobstein made the observation that the ureteric bud induced the nephrogenic mesenchyme to undergo tubulogenesis. Since that discovery, scientists have attempted to characterize the molecular nature of the inducer. To date, no single molecule that is both necessary and sufficient for nephric induction has been identified. Because of recent insights regarding the role of several secreted molecules in tubulogenesis, it has become necessary to revise the classic model of metanephric induction. The studies of the classic ureteric inducer performed to date have most likely been characterizations of a mesenchyme-specific inducer, Wnt-4, and its role in tubulogenesis. Ureteric induction most likely involves a series of distinct events that provide proliferative, survival, and condensation signals to the mesenchyme, integrating the growth of the ureteric system with tubulogenesis.

The developmental biologic processes of the kidney have been the subject of intense study for more than 100 yr (for review, see reference (1). All three vertebrate kidney types (pronephros, mesonephros, and metanephros) are derivatives of a region of the embryo known as the intermediate mesoderm. In mice, a portion of the mesonephric duct, known as the metanephric bud, branches dorsally approximately 10.5 d after coitus and invades the caudal-most portion of the nephrogenic mesenchyme. The bud branches several times while growing peripherally and eventually forms the collecting duct system of the mature kidney. Shortly after invasion by the bud, the mesenchyme juxtaposed to the bud condenses, undergoes an epithelial transformation, and fuses with the duct, forming the metanephric tubule. The tubule elongates and proceeds through a series of intermediate forms, known as the comma- and S-shaped bodies, before ultimately forming the mature renal tubule. The proximal-most region of the tubule forms a specialized structure, *i.e.*, the glomerulus, which is vascularized and is the primary filtration apparatus of the organ. Mature murine kidneys consist of approximately 0.5 million precisely arranged functional units, or nephrons, connected to the collecting duct system. Because of the importance of the kidney in maintaining proper blood chemistry values, the correct arrangement and number of nephrons are essential.

It has been nearly 50 yr since Clifford Grobstein discovered that interactions between the ureteric bud and the adjacent mesenchyme affect the normal development of the metanephric kidney (2–6). Grobstein revealed that, if the ureteric bud is separated from the metanephric mesenchyme at an early stage of development, then the mesenchyme fails to undergo tubulogenesis. The conclusion drawn from this discovery was that the ureteric bud induces tubulogenesis within the surrounding mesenchyme. During further investigation, it was discovered that a number of tissues, including, most notably, a dorsal portion of the embryonic spinal cord, are able to substitute for the ureter in this inductive interaction. Although the spinal cord certainly plays no role in the normal induction of the mesenchyme, it was assumed that this functional mimicry is attributable to the expression of the endogenous inducer within the spinal cord tissue. In fact, the spinal cord seems to be a far more effective inducer than the ureter itself and, since this discovery, it has been used in place of the ureter in the majority of studies on metanephric induction. For example, observations that the inductive event could take place across a filter (suggesting that the inducer was a secreted molecule) but required a large enough pore size to allow cell/cell contact (suggesting that the secreted molecule was closely associated with the cell membrane) were made using spinal cord as the inductive source.

In recent years, developmental biologists have sought the molecules responsible for a number of inductive signals that were first identified embryologically, including the ureteric inducer. On the basis of the initial characterizations made by Grobstein, candidate molecules for the kidney inducer should satisfy a number of criteria (7). First, and most important, a candidate molecule must be expressed in the ureteric bud at the time of induction, *i.e.*, 10.5 d after coitus in mice. Second, a candidate molecule must be a secreted factor that is closely associated with the cell membrane. Finally, if the molecule is truly involved in tubule induction, then it must be both necessary and sufficient for normal induction.

This model makes the assumption that there exists a single inducing molecule, which is the simplest hypothesis in the absence of contradicting data. However, to date, no single molecule that satisfies all of these criteria has been identified. Several secreted molecules, including members of the bone morphogenetic protein, fibroblast growth factor (FGF), insulin-like growth factor, and Wnt families of growth factors, are

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expressed in the invading ureteric bud; however, none of them are capable of inducing tubulogenesis and thus cannot be the classic inducer (for review, see reference 8). Although it was recently demonstrated that members of the *wingless/int* family of secreted glycoproteins are capable of inducing tubulogenesis *in vivo* (9), to date only one Wnt (Wnt-11) that is expressed in the tip of the ureteric bud during the inductive phase has been identified (10). Unfortunately, as discussed below, this molecule is not sufficient to induce tubulogenesis *in vitro*. If a single inductive factor exists, it cannot be one of the already characterized Wnt proteins. Furthermore, current data suggest that the Wnt signal originates not from the ureter but rather from the metanephric mesenchyme (11). In light of these data, it seems appropriate to reevaluate the nature of the metanephric inducer and the role of secreted proteins in the process of induction.

Role of Wnt Proteins in Kidney Development

The Wnt proteins have been demonstrated to play essential roles in a number of developmental processes in a wide range of organisms (for review, see reference (12) or www.stanford.edu/~musse/pathways/allcomp.html). The discovery that cell lines expressing Wnt-1 are capable of inducing metanephric mesenchyme to undergo tubulogenesis (9) led to the hypothesis that Wnt proteins may function as the classic metanephric inducer. In fact, the spinal cord, which is capable of substituting for the endogenous inducer, expresses several Wnt proteins, providing further circumstantial evidence that this important family of molecules may be involved in metanephric induction. Because Wnt-1 is not expressed in developing kidneys, it has been hypothesized that this molecule mimics another Wnt in *in vitro* experiments. To date, four Wnt proteins that are expressed in the developing metanephros have been identified, *i.e.*, Wnt-4, -6, -7b, and -11 (10). Because Wnt-6 is expressed at extremely low levels and is thought not to play a major role in kidney development, it is not further discussed. We concentrate on the prospective roles of Wnt-4, -7b, and -11 in induction and later nephric development.

Wnt-4

The first Wnt shown to be involved in kidney development was Wnt-4 (13). Wnt-4 is expressed in the aggregating metanephric mesenchyme at 11.5 d after coitus, shortly after ureteric bud invasion. Targeted ablation of this gene results in embryos with severely hypoplastic kidneys. In the absence of Wnt-4, the metanephric mesenchyme condenses normally but fails to aggregate into pretubule clusters and undergo tubulogenesis. Morphologic and molecular marker analyses suggest that Wnt-4 is involved in the transition of tubular mesenchyme to epithelium. Interestingly, the ureteric epithelium of mutant kidneys undergoes several rounds of branching, suggesting that tubulogenesis is not required for this process. The expression patterns and phenotypes of Wnt-4 mutants suggest that Wnt-4 is a mesenchymal factor that acts downstream of the initial inductive events. Therefore, Wnt-4 cannot be the ureteric inducer.

Wnt-7b

A second family member that is expressed in the developing metanephros is Wnt-7b (10). Unlike Wnt-4, Wnt-7b is expressed within the epithelium of the ureteric bud. However, the time at which Wnt-7b mRNA can first be detected within the ureteric epithelium (13.5 d after coitus) is not consistent with this molecule playing a role in induction. Furthermore, Wnt-7b mRNA is excluded from the tips of the ureter, the region thought to possess inductive properties, further diminishing the possibility of a role for this molecule in the inductive process. Preliminary genetic analyses suggest that Wnt-7b plays a role in duct maintenance or growth (Carroll, Pepicelli, & McMahon, unpublished observations), rather than induction.

Wnt-11

A search for Wnt genes expressed in the developing kidney revealed the previously uncharacterized Wnt-11 (10). The expression pattern of this gene was particularly intriguing to nephrologists. Wnt-11 is expressed within the mesonephric duct just before metanephric induction. As the ureteric bud invades the metanephric blastema, Wnt-11 expression intensifies within the leading edge of the ureteric bud. As the ureter branches, Wnt-11 expression is maintained at the tips of each branch but is lost in the intervening epithelium. Wnt-11 continues to be expressed at the tips of the ureter as they move toward the cortex of the developing kidney, which is a region of continuing induction.

Wnt-11 expression closely matches that expected for a molecule involved in tubule induction. However, when cell lines expressing Wnt-11 were co-cultured with metanephric mesenchyme, they failed to induce tubulogenesis (11). Wnt-11 therefore fails to satisfy at least one of the criteria (sufficiency) for a metanephric inducer. It seems unlikely that this molecule plays a role in induction. Quite surprisingly, in this same assay it was found that cell lines expressing Wnt-4 were capable of inducing tubulogenesis. This result is surprising in light of the fact that Wnt-4 was previously shown to act downstream of the ureteric signal. Furthermore, it has been shown that metanephric mesenchyme itself is not capable of inducing tubulogenesis (so-called homeogenetic induction) (14). The fact that a gene that is expressed within the metanephric condensations and acts downstream of the ureteric signal is capable of inducing tubulogenesis is quite unexpected.

Signal Relay in Tubulogenesis

As mentioned above, nearly all of the characterizations of metanephric induction have been performed using spinal cord as the inductive tissue, under the assumption that this tissue mimics the endogenous inducer. Because the Wnt molecules behave identically to the spinal cord in this *in vitro* assay, it has also been assumed that they mimic the ureteric signal. However, the ability of Wnt-4 to induce tubulogenesis necessitates a reevaluation of these findings.

If the Wnt molecules truly mimic the ureteric inducer, then co-culture of a Wnt-expressing cell line with Wnt-4 mutant mesenchyme should not induce tubulogenesis (because Wnt-4 has been shown to produce its effect downstream of the ure-

teric signal). Quite surprisingly, this was not the case. Several Wnt-expressing cell lines could induce tubulogenesis in Wnt-4 mutant mesenchyme, suggesting that the Wnt proteins produce their effects at the level of or downstream of Wnt-4 (11). Therefore, it seems that the Wnt proteins do not mimic the classic inductive signal at all. The characteristics of the classic inducer were defined using spinal cord as the inductive tissue. The spinal cord, like the Wnt proteins, was assumed to mimic the endogenous ureteric signal. The ability of the spinal cord to induce tubulogenesis might be attributable to the fact that it expresses several Wnt proteins (including Wnt-4), rather than the possibility that it expresses the endogenous inducer. To investigate this possibility, the inductive properties of the spinal cord were reexamined (11). If the spinal cord mimics a ureteric signal, its inductive abilities should be blocked by Wnt-4 mutant mesenchyme. However, if the spinal cord is simply mimicking the Wnt signal, then it should be able to induce tubulogenesis in Wnt-4 mutant mesenchyme. Rather surprisingly, the latter hypothesis seems to be correct. Spinal cord is capable of inducing tubulogenesis just as well in Wnt-4 mutant mesenchyme as in wild-type mesenchyme. This finding strongly suggests that the spinal cord signal is acting at the level of or downstream of Wnt-4 within the metanephric mesenchyme. This further suggests that most studies of the nature of the metanephric inducer that have been performed to date have actually characterized a mesenchymal inducer, Wnt-4. Metanephric induction seems to involve a series of events that originates in the ureter but includes mesenchyme-specific signals.

Ureteric Inducer 2000

The data reviewed here suggest that much of what we know (or thought we knew) regarding the nature of the ureteric inducer is probably incorrect. The studies performed to date have most likely characterized Wnt-4 and its role in tubulogenesis. These recent discoveries present nephrologists with a number of unanswered questions. What do we know regarding the molecular nature of the ureteric signal? Because we must ignore much of the previous data (because it was obtained using spinal cord), the only fact we know definitely is that the ureter is necessary for tubulogenesis. It remains unclear whether the ureter itself actually induces tubulogenesis. There are several alternative explanations for the lack of tubulogenesis after removal of the ureter. For example, the ureter could provide survival and/or proliferative signals to the metanephric mesenchyme (dead cells do not form tubules). This is supported by the observations that mesenchyme cultured in the absence of an inducer undergoes apoptosis (15) and some signals produced by the ureter, including bone morphogenetic protein-7, promote survival and growth of the mesenchyme but do not induce tubulogenesis (16,17). However, other factors produced by the ureter, including β-FGF (18), cause cultured metanephric mesenchyme to condense, suggesting that this structure provides more than just survival or proliferative signals. These data suggest that metanephric induction is not a single event. Further support for this notion is derived from several recent studies. It has been demonstrated that an unidentified factor (or factors) from a ureteric bud cell line, in combination with β-FGF and transforming growth factor-α (TGF-α), supports full differentiation of cultured metanephric mesenchyme (19,20). Recently, Barasch *et al.* (21) demonstrated that leukemia inhibitory factor (LIF), which by itself is unable to induce tubulogenesis, can induce full differentiation when supplemented with β-FGF and TGF-α, suggesting that it may be the unidentified ureteric factor. Interestingly, although LIF is expressed in the ureter, it seems to act downstream of the primary inductive signal, inasmuch as markers of the initial inductive response (Pax-2 and Wnt-4) are expressed in cultured mesenchyme in its absence (21). This observation correlates well with the fact that β-FGF by itself can cause condensation but not tubulogenesis of metanephric mesenchyme (18). However, it is surprising that the expression of Wnt-4 in mesenchyme cultured with β-FGF and TGF-α does not lead to tubulogenesis, as would be expected from the *in vitro* experiments. There are clearly regulatory events that we do not now understand, and further investigation is needed.

Conclusion

Currently available data support a multifactorial process for metanephric induction. Tubulogenesis most likely requires multiple sequential inductive signals from the ureter, in combination with feedback from the mesenchyme to the ureter (Figure 1). After the initial inductive event (possibly involving β-FGF), the mesenchyme is signaled to condense. Further differentiation (tubulogenesis) requires additional signals, which, at least *in vitro*, can be provided by the LIF molecule. Placing Wnt-4 within this system is more difficult. The experiments by Barasch *et al.* (21) suggest that Wnt-4 is downstream of the FGF-like signal but upstream of LIF. However, at least *in vitro*, Wnt-4 alone is sufficient to induce tubulogenesis in the absence of additional ureteric factors, seemingly in contradiction to these results. Further investigation is certainly needed to establish the organizational relationships of these molecules during kidney development. It would be interesting to test the ability of Wnt-4 to induce tubulogenesis in LIF mutant mesenchyme. Unfortunately, neither LIF nor its receptors manifest a severe kidney phenotype when mutated (perhaps because of molecular redundancy), precluding these experiments. However, assessments of the ability of LIF to induce tubulogenesis in Wnt-4 mutant mesenchyme can be performed and should help us to better understand this series of events.

Regardless of how these signals relate to each other, it seems quite clear that multiple signals from the ureteric bud and the metanephric mesenchyme cooperate to integrate the survival, proliferation, and condensation of the metanephric blastema with ureteric branching. This sequence leads to a tightly regulated and precisely controlled process of nephric morphogenesis. With increased understanding, we should be able to differentiate each of these events at both the molecular and morphologic levels. The next several years should provide us with evidence that will further clarify these processes. Whatever the future brings, it seems certain that the classic one-signal model for metanephric induction is greatly oversimplified. The future holds great promise for the discovery of unique

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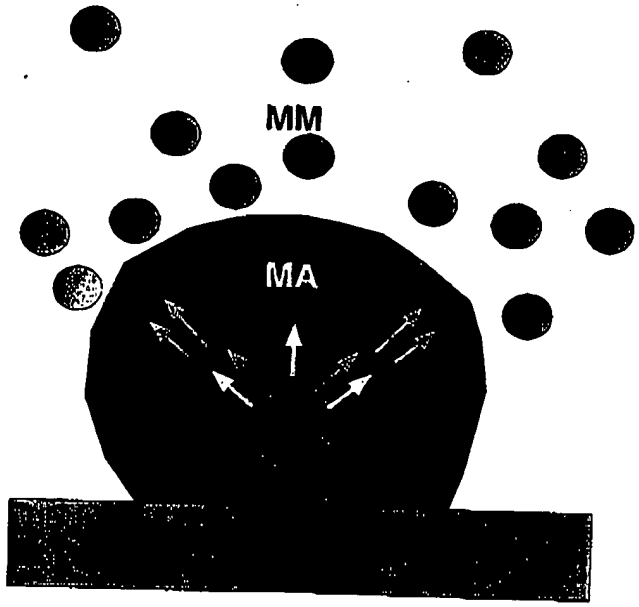

Figure 1. Current model for induction of the metanephric mesenchyme. The ureteric bud (UB) secretes several initial signals that lead to the survival, proliferation, and aggregation of the metanephric mesenchyme (MM). The initial signaling events activate Wnt-4 expression within the mesenchymal aggregates (MA). Wnt-4 acts as a mesenchyme-specific inducer of tubulogenesis. Additional signaling from the ureter seems to be required for morphogenesis of the tubules. Leukemia inhibitory factor (LIF) may represent this additional factor. bFGF, basic fibroblast growth factor; TGFa, transforming growth factor α; BMP's, bone morphogenetic proteins; FGF's, fibroblast growth factors.

factors and further understanding of previously identified factors in ureteric and mesenchymal signaling. With the precise roles of several Wnt molecules still unknown, it seems likely that this important family of molecules will be demonstrated to play additional roles in the complex series of events that leads to the development of the metanephros.

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Wnt genes and vertebrate development

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A variety of experimental approaches have underscored the critical role played by secreted polypeptide factors, such as those encoded by members of the *Wnt* gene family, in many aspects of vertebrate embryogenesis. Recent papers have revealed restricted patterns of *Wnt* gene expression that delineate important subdivisions within the early forebrain and spinal cord, demonstrated that *Wnt* gene products can regulate mesoderm formation and gastrulation, and investigated how Wnt protein signaling may affect cell adhesion.

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Introduction

Communication between cells is necessary to achieve the proper organization of the growing vertebrate embryo. Intercellular signals are often transmitted by secreted polypeptides, such as members of the transforming growth factor (TGF)-β, fibroblast growth factor (FGF), and Wnt families. Initial interest in the function of vertebrate *Wnt* genes in embryogenesis centered on the role of *Wnt*-1 in midbrain and hindbrain development [1–3]. The cloning of additional *Wnt* genes has expanded the domains of Wnt signaling to include other regions of the CNS and other processes during embryogenesis. This review examines the possible regulation of forebrain and spinal cord development by *Wnt* genes and the role played by Wnt proteins during mesoderm formation and gastrulation. We also review possible components of Wnt signaling pathways that may generate alterations in cell adhesion properties.

Wnt genes and CNS development

A growing number of *in situ* hybridization studies have shown that many *Wnt* genes are expressed in the embryonic CNS and have hinted that Wnt signaling plays an important role throughout the early neural tube [4–6,7•]. The timing of initial *Wnt* gene expression in the CNS [8.0–9.5 days post-coitum (dpc) in the mouse] suggests that these genes could assist in the regional specification of the neural tube. Perhaps most intriguing are the patterns of *Wnt* gene expression in the forebrain and spinal cord.

Wnt gene expression in the spinal cord exhibits striking restrictions along the dorsal/ventral axis prior to any differentiation of neurons, *Wnt*-1, *Wnt*-3, and *Wnt*-3a expression along the dorsal midline being one of the earliest markers of dorsal patterning in the spinal cord [3,4,6,7•,8]. Neural crest cell precursors also migrate from the dorsal midline at this time, and strong β-galactosidase activity is seen in neural crest cells whose progenitors expressed a *lacZ* transgene driven by *Wnt*-1 regulatory elements [9•]. *Wnt*-4 is expressed in a broad dorsal domain that probably includes the presumptive alar plate, which is destined to produce sensory interneurons [7•]. *Wnt*-7a and *Wnt*-7b expression is located ventromedially in the 9.5 dpc mouse spinal cord [7•]. Most ventrally, *Wnt*-4 is also expressed in the floor plate [7•,10], a region known to influence patterning of the spinal cord [11]. These expression patterns suggest that *Wnt* genes could reinforce dorsal/ventral polarities that have been established by molecules such as dorsalin-1 and Sonic hedgehog [12••–16•]. It seems even more likely that Wnt proteins may be regulating patterns of cell proliferation in the spinal cord; ectopic expression of *Wnt*-1 under the control of a *Hoxb*-4 enhancer severely disrupts normal spinal cord morphology, probably as a consequence of overproliferation, but does not appear to have a primary effect on dorsal/ventral patterning [17•].

At least seven mouse *Wnt* genes are expressed in the forebrain by 9.5 dpc [3,7•,18]. *Wnt*-5a and *Wnt*-7a are expressed in overlapping domains in the ventral and lateral diencephalon, whereas *Wnt*-1, *Wnt*-3, *Wnt*-3a, and *Wnt*-4 mRNAs are present in the dorsal diencephalon. *Wnt*-7b is expressed throughout much of the diencephalon, the optic stalks, and the dorsal optic vesicle. *Wnt*-3a and *Wnt*-7b expression extends into the telencephalon, *Wnt*-3a along the dorsal midline, and *Wnt*-7b in a broad dorsal band. Thus, *Wnt* genes have the potential to be important regulators of early forebrain development.

Abbreviations

CNS—central nervous system; dpc—days post-coitum; *en—engrailed*; FGF—fibroblast growth factor; *hh—hedgehog*; TGF—transforming growth factor; *wg—wingless*.

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The vertebrate hindbrain appears to be composed of segmental units or rhombomeres [19], and accumulating evidence suggests that part of the embryonic forebrain may be subdivided in a similar fashion [20,21•,22•]. Patterns of gene expression generally coincide with, and often predict, the forebrain partitions [18,22•]. *Wnt* genes provide particularly good examples of restricted gene expression in the forebrain prior to the appearance of overt morphological subdivisions. For example, a triangular patch of *Wnt*-3a expression in the diencephalon appears to correspond to the presumptive pretectum or synencephalon [7•], and a domain of *Wnt*-3 expression eventually becomes the dorsal thalamus [18,22•]. Although not so obviously expressed in discrete patches, *Wnt*-7b expression is strongly up-regulated in the aforementioned *Wnt*-3a domain in the diencephalon [7•]. Apart from their expression in putative forebrain neuromeres, *Wnt* mRNAs clearly demarcate dorsal/ventral zones that may represent subdivisions comparable to the roof, alar, and basal plates in the more caudal neural tube [7•].

The past year has also witnessed the first indication that *Wnt* genes could be involved in hindbrain segmentation. *Cwnt*-8c expression in the chick hindbrain becomes localized to presumptive rhombomere 4 prior to the formation of rhombomere boundaries and to the restriction of *Hoxb*-1 expression to the same region [23•]. Interestingly, rhombomeres 3 and 5 express a number of specific genes (e.g. *Krox*-20 and *msx*-2) and undergo unique patterns of cell death that appear to be controlled by neighboring rhombomeres [24,25]. It will be intriguing to determine whether *Cwnt*-8c can regulate these processes.

Axis formation and gastrulation

The possible involvement of *Wnt* genes in axis formation was suggested initially by the ectopic expression of *Wnt*-1 in *Xenopus* embryos, which leads to a duplication of the primary embryonic axis [26]. Subsequently, other *Wnt* genes have proved capable of generating axial duplications in various ectopic expression assays [8,27,28]. The main difficulty in assigning a role to Wnt proteins in the normal process of axis formation has been discovering endogenous *Wnt* genes that are expressed at the proper time and place.

Current models of axial patterning, derived primarily from *Xenopus* experiments, suggest at least three possible functions for Wnt proteins [29,30]. Firstly, they could assist in the initial establishment of dorsal versus ventral sides of the embryo soon after fertilization and be components of dorsalizing (Nieuwkoop center) or ventralizing centers of mesoderm induction. Secondly, they could act as parts of the dorsal (Spemann organizer) or ventral signals that modify the character of pre-existing mesoderm. Thirdly, they could help produce specified mesoderm as the primary axis information is 'read out' during gastrulation. Although *Wnt* products may participate in the first of these processes, no strong candidates exist among the well characterized *Wnt* genes. In contrast, good evidence points to Wnt proteins affecting the second and third processes.

Xwnt-8 and Xwnt-11 are capable of modifying the character of mesoderm that has been induced by other signals. Although Xwnt-8 possesses dorsalizing activity when expressed ectopically by the 32-cell stage of *Xenopus* development [27,28], it is normally expressed on the ventral side of the embryo at late blastula and gastrula stages [31]. Christian and Moon [32•] resolved this apparent paradox by expressing *Xwnt*-8 ectopically at the time that it is normally transcribed. Xwnt-8 does possess ventralizing activity when expressed dorsally at this later time and is, therefore, a good candidate for a ventralizing signal.

Conversely, Xwnt-11 is capable of dorsalizing previously induced mesoderm [33•]. *Xwnt*-11 mRNA is localized to the vegetal cortex and cytoplasm of the *Xenopus* oocyte and egg. By the late blastula stage, *Xwnt*-11 is strongly expressed in the marginal zone, with the highest transcript levels being present dorsally. Injected *Xwnt*-11 mRNA can partially rescue UV-ventralized embryos. Both Xwnt-11 and Xwnt-8 must be modifying the character of pre-existing mesoderm in these experiments, because they are incapable of directly inducing mesoderm on their own.

Wnt proteins probably act as important mediators when the information for the primary embryonic axis is deciphered during gastrulation. The most direct evidence for the participation of Wnt proteins in gastrulation comes from a targeted mutation in the mouse *Wnt*-3a gene [34•]. *Wnt*-3a expression is not seen when gastrulation begins at 6.5 dpc, being first detected at 7.0–7.5 dpc throughout the primitive streak. Most importantly, *Wnt*-3a is the only known *Wnt* gene to be expressed in regions of the primitive streak fated to generate dorsal (somitic) mesoderm at this time. *Wnt*-3a mutants exhibit a severe truncation of the body axis caudal to the forelimbs. Dorsal mesoderm is particularly affected, as caudal somites are missing. In the most caudal regions, the notochord is disrupted and no tailbud forms. The timing of *Wnt*-3a expression in the primitive streak appears to be critical, as somites anterior to the forelimbs are unaffected in the mutants and may be specified prior to *Wnt*-3a activation. Thus, *Wnt*-3a expression is required for the production of somitic mesoderm and also appears to be necessary for the generation of all new embryonic mesoderm by late primitive streak stages.

Wnt-5a and *Wnt*-5b are also expressed in the primitive streak during gastrulation in the mouse, although primarily in cells with extra-embryonic fates [34•]. *Xenopus Xwnt*-5a is expressed throughout embryonic development and generates a complex set of head and tail malformations when overexpressed [35•]. Overexpression of *Xwnt*-5a does not induce mesoderm or change the type

of pre-existing mesoderm, but can inhibit the elongation of blastula cap explants. This suggests that Xwnt-5a may affect the normal morphogenetic movements that occur during gastrulation.

The chicken *Cwnt*-8c gene is expressed in the posterior marginal zone prior to gastrulation and later in the primitive streak and Hensen's node [23•]. Injection of *Cwnt*-8c mRNA into *Xenopus* embryos can dorsalize mesoderm and generate axial duplications. Cwnt-8c could, therefore, function both as a component of the organizer region and later in gastrulation.

We now have a rapidly increasing number of *Wnt* genes that truly appear to play a part in axis formation and gastrulation. No endogenous candidates for mesoderm induction or initial patterning have yet been found, but Wnt proteins capable of modifying pre-existing mesoderm (Xwnt-8, Xwnt-11, and Cwnt-8c) and affecting gastrulation (Wnt-3a and possibly Wnt-5a, Wnt-5b, and Cwnt-8c) are now in hand. How these Wnt proteins cooperate with molecules such as activins, FGFs, Brachyury, noggin, and goosecoid in axial patterning is an active area of research that will probably provide useful insights into the mechanisms of Wnt signaling [32•,36–39].

Wnt signaling pathways

We still know little about the identity of molecules that function upstream and downstream of *Wnt* genes in vertebrate development. Most of our knowledge about Wnt signaling has been gathered from *Drosophila* genetics. Interactions between cells expressing *wingless* (*wg*) and *engrailed* (*en*), situated on either side of the parasegmental boundary, are necessary to establish the segmental pattern of the *Drosophila* embryo (reviewed in [40]). Studies of midbrain and hindbrain development in *Wnt*-1 mutant mice suggest that the functional connection between *Wnt* and *engrailed* genes may be conserved in vertebrates [3]. Will the homologs of other *Drosophila* genes required for *wg* function also be components of vertebrate *Wnt* signaling pathways?

The *hedgehog* (*hh*) gene product signals from *en*-expressing cells back to *wg*-expressing cells during segmentation in *Drosophila* embryos [41]. The initial characterization of vertebrate *hh* genes has indicated that they may have important roles in limb and neural tube patterning [12•–15•]. The expression patterns of published vertebrate *hh* genes, however, do not strongly correlate with *Wnt* gene expression in an obvious fashion. Thus, no vertebrate *Wnt–hh* connection has been established at this time.

The *armadillo* gene is required for the interpretation of the wg signal in *en*-expressing cells [42]. The *armadillo* product is related to the cytoskeletal-associated proteins β-catenin and plakoglobin, which modulate the function of the cadherin cell adhesi n m lecules and participate in the formation of adherens junctions [43,44]. These findings suggest a means by which Wnt signaling could affect cell adhesion and several recent papers have offered hints about the possibly complex interplay between Wnt proteins and catenins in vertebrates.

Expression of *Wnt*-1 in mouse C57MG mammary epithelial cells or AtT20 neuroendocrine cells increases the levels of β-catenin and plakoglobin proteins [45•]. The consequences include stabilized binding of β-catenin to cadherin, increased stability of cell surface cadherin, and stronger cell–cell adhesion. Similarly, *Wnt*-1 expression in rat PC12 cells produces increased levels of plakoglobin (but not β-catenin) and E-cadherin proteins and strengthens calcium-dependent cell adhesion [46•]. This seemingly clear picture, whereby increased *Wnt* expression leads to increased cell adhesion through a β-catenin or plakoglobin intermediary, becomes cloudier when *in vivo* experiments are examined.

Some *Xenopus* experiments support the relatively simple model proposed above. One consequence of ectopic *Wnt*-1 expression is an increase in gap junctional communication, a process that probably requires increased cell adhesion [47]. Overexpression of *Wnt*-5a seems to increase cell adhesion and reduce cell mixing during gastrulation [35•]. These responses to *Wnt*-5a are similar to those produced by the overexpression of N-cadherin [48]. However, ectopic expression of *Wnt*-1 or *Wnt*-5a does not lead to reproducible changes in the levels of β-catenin and plakoglobin RNA or protein [49]. On the other hand, when antibodies to β-catenin are injected into *Xenopus* embryos, the resulting axial duplications are strongly reminiscent of those induced by ectopically expressed *Wnt*-1 or *Xwnt*-8 [50•]. Somewhat surprisingly, no obvious changes in cell adhesion were seen in this set of experimental embryos.

Alterations in catenin and cadherin expression in *Wnt*-1 mutant mice provide additional surprises (K Shimamura, S Hirano, A McMahon, M Takeichi, unpublished data). *Wnt*-1-expressing cells are normally on the periphery of regions that express E-cadherin. In *Wnt*-1 mutant embryos, E-cadherin is strongly up-regulated in cells that would normally express *Wnt*-1, suggesting that Wnt-1 can suppress E-cadherin expression. No detectable changes in plakoglobin or β-catenin expression are evident in *Wnt*-1 mutants. However, a down-regulation of αN-catenin expression is seen in the dorsal midline cells that normally express *Wnt*-1. This is the first indication of a possible connection between Wnt proteins and α-catenins.

Taken together, these experiments indicate that, depending upon the particular cellular context, Wnt proteins may be able to alter the expression of various catenins and cadherins in more than one fashion. Changes in the intracellular distribution of armadillo protein in response to the wg signal point to an additional level of regulation in the Wnt signaling pathways [42,51]. Recent findings have also revealed that a domain

of the β-catenin protein is shared with a guanine nucleotide exchange protein and interacts with the tumor suppressor gene associated with adenomatous polyposis coli (APC) [52–54]. Consequently, signaling through β-catenin is likely to involve more than simple changes in cell adhesion properties.

Moving upstream, little is known about the regulation of *Wnt* gene expression. The initial characterization of regulatory elements at the 3′ end of the mouse *Wnt*-1 gene opens the door to discovering transcription factors governing *Wnt* gene expression [9•]. The expression of *wg* in *Drosophila* embryos is regulated by transcription factors such as paired and gooseberry [55,56], whose vertebrate homologs, the *Pax* genes, are expressed in many of the same areas as *Wnt* genes [57]. However, direct functional connections between these overlapping expression patterns remain to be established.

Conclusions

Following the cloning and initial characterization of *Wnt* genes from many vertebrate species, the first functional studies have demonstrated roles for *Wnt* genes in gastrulation and CNS development. In addition to further research along those lines, areas for future exploration include interactions between Wnt proteins and members of other families of regulatory molecules, possible functional redundancy between *Wnt* family members, *Wnt* gene function in organogenesis, and evolutionary comparisions of *Wnt* gene function in different species. Most of the Wnt signaling pathway(s) remains to be uncovered, particularly the ever elusive Wnt receptor(s). The uncertainties surrounding virtually all aspects of *Wnt* gene function guarantee some interesting surprises in the future.

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